

Research Article

Comparison of Newcastle disease vaccination programs: Influence of vaccine type, delivery method, and timing on immune response in broilers

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ABSTRACT

Newcastle disease (ND) remains one of the most important viral diseases affecting poultry health and productivity worldwide, causing substantial economic losses. Although vaccination is a cornerstone of ND control, differences in vaccine type, administration method, and timing may influence the magnitude of the humoral antibody response. This study evaluated different ND vaccination programs in broiler chickens under controlled experimental conditions by comparing strategies that varied in initiation age, number of administrations, and administration route, including spray, injection, and eye-drop methods. The primary outcome was the hemagglutination

inhibition (HI) antibody titer measured at Days 35 and 42. The observed data showed that programs initiated on the first day of life were associated with higher HI antibody titers in several protocol comparisons. The highest observed mean was recorded in a protocol that included spray administration; however, because the protocols also differed in timing, number of administrations, and vaccine combinations, the independent contribution of route could not be isolated. Differences were also observed between protocols using single ND vaccines and combined ND–avian influenza (AI) vaccination. These findings describe humoral antibody responses under the conditions of this controlled experiment. Because the study did not include a challenge experiment and the protocols were not independently replicated at the pen level, the results do not establish protective efficacy or confirmatory superiority of one vaccination program.

Abbreviations

APMV-1: Avian Paramyxovirus Type 1; HI: Hemagglutination Inhibition; ND: Newcastle Disease; NDV: Newcastle Disease Virus; VN: Virus Neutralization

Introduction

ND is a highly contagious and economically important viral infection of poultry that continues to threaten global poultry production. It is caused by virulent strains of Avian Paramyxovirus type 1 (APMV-1), classified under the genus Avulavirus, family Paramyxoviridae, and order Mononegavirales [1,2]. The disease presents with a wide spectrum of clinical manifestations—including respiratory, gastrointestinal, and neurological signs—ranging from mild to severe forms with high mortality [3]. ND remains endemic in many countries, including Iran, leading to major economic losses due to reduced productivity, mortality, and control expenses. Beyond chickens, outbreaks have also been reported in ostriches [4] and Japanese quails [5], indicating its wide host range and zoonotic potential.

Effective ND control relies primarily on strict biosecurity and vaccination programs [6]. Since its introduction in the 1940s, vaccination has greatly reduced the severity and frequency of ND outbreaks [7]. Nevertheless, ND continues to occur frequently in developing countries, where it remains a critical concern for poultry-associated food security, despite the extensive application of currently available vaccines [8,9]. The highly contagious nature of NDV and the ability of wild birds to carry the virus asymptotically mean that outbreaks can occur wherever poultry are raised. Disease prevalence, circulating genotypes, and management practices vary across countries and farms, emphasizing the need for fit-for-purpose application of ND vaccines and development of next-generation ND vaccines [8].

Both live attenuated and inactivated vaccines are employed to reduce clinical signs and mortality; however, factors such as incomplete prevention of viral shedding, antigenic variation, and stress-induced immunosuppression can compromise vaccine efficacy [10]. Recent studies highlight the potential of locally developed inactivated ND vaccines in effective disease control. For instance, an inactivated vaccine derived from locally circulating genotype-VII.2 NDV isolates

showed promise in controlling ND in commercial poultry populations in Bangladesh [11]. Similarly, in Ethiopia, genotype VII.1.1 NDV is widely distributed, and outbreaks in vaccinated flocks are often associated with management failures or immunosuppression from other infections, rather than antigenic mismatch, as these viruses exhibited similar antigenicity to the HB1 vaccine in VN tests [12]. Additionally, experimental studies have shown that administration of mismatched ND vaccines, such as LaSota and Clone.12IR, can still confer protection against genotype VII virulent NDV and reduce viral shedding, providing important guidance for designing effective vaccination strategies in poultry farms [8].

Various routes are available for vaccine administration, including eye drops, drinking water, sprays, and injections, each with distinct advantages depending on flock size, labor resources, and desired immune response. Eye-drop and spray vaccination stimulate the Harderian gland, enhancing mucosal immunity [13,14]. Spray vaccination has been shown to induce stronger and longer-lasting immune responses than drinking-water methods [15,16], although it may sometimes cause respiratory irritation in young chicks [17]. Immunity against ND is mediated through circulating antibodies in the blood, secretory antibodies providing mucosal immunity, and cell-mediated immune responses. Live ND vaccines contain live viruses capable of infecting cells and mimicking natural infection, thus stimulating all three immune response types. In contrast, killed vaccines, rendered non-infectious by chemical agents, radiation, or heat, only induce humoral immune responses [18].

Vaccine efficacy is also influenced by strain, dosage, and the combination of live and inactivated vaccines. The LaSota strain is often reported to induce higher antibody titers than B1 and Mukteswar strains [19,20]. Combining live vaccines with inactivated oil-based vaccines has proven effective in areas with high ND prevalence, enhancing antibody titers and providing prolonged immunity. Recent research also suggests that protocols starting with a spray of live

vaccine on day 1, followed by inactivated vaccine injection and oral live vaccine doses, provide superior protection, while inactivated vaccines administered in the first week may be less effective [20].

Routine serological monitoring using hemagglutination inhibition (HI) tests is essential to assess flock immunity, evaluate vaccination effectiveness, detect immunity gaps, and identify active infections [3]. Despite these measures, variation in immune responses among flocks suggests that many vaccination programs remain suboptimal under field conditions. Optimizing vaccination programs aims to minimize disease risk, maximize production efficiency, and balance cost and potential negative effects on growth [3].

Therefore, the objective of the present study was to describe and compare HI antibody responses associated with commonly used ND vaccination protocols under controlled experimental conditions. The evaluated protocols differed in vaccine formulation, age at initial vaccination, administration route, and number of administrations. Because these features were not independently varied in a fully replicated factorial design, the study was intended to compare the observed responses among complete vaccination protocols rather than to estimate the isolated causal effect of each individual component.

Results

The observed HI antibody titers at 35 and 42 days of age across the evaluated vaccination protocols are summarized in Table 3. Because each protocol was represented by a single subgroup, between-protocol differences are presented descriptively and should not be interpreted as confirmatory treatment effects.

Vaccination timing

Among the evaluated protocols, subgroup F2 exhibited the highest observed mean HI antibody titers, reaching 5.45 ± 0.170 at Day 35 and 5.95 ± 0.256 at Day 42. In contrast, subgroup B4 showed one of the lower observed mean titers (3.25 ± 0.310) at Day 35. These are descriptive comparisons among the protocols evaluated in this experiment; the design does not permit a confirmatory conclusion that vaccination timing alone caused the observed differences.

Vaccine type

Differences were observed between protocols involving a single ND vaccine and those involving combined ND + AI vaccination. Subgroup A2 (single ND vaccine) had an observed mean HI antibody titer of 5.30 ± 0.325 at Day 35, whereas subgroup B2 (combined vaccination) had a mean of 3.85 ± 0.254 at the same age. Because the protocols differed in other features as well, this comparison cannot establish an independent effect of vaccine formulation or antigen combination.

Vaccine dose frequency

Observed HI antibody titers varied among vaccination programs that differed in the number of vaccine administrations. For example, subgroup F1, which received five administrations, had a mean HI antibody titer of 5.05 ± 0.346 at Day 42, compared with 5.00 ± 0.293 in subgroup F4, which received fewer administrations. This small numerical difference should not be interpreted as evidence of an overall effect of vaccination frequency, particularly because the protocols differed in other aspects and each protocol lacked independent pen-level replication.

Administration route

Observed HI antibody titers varied among vaccination programs using different administration routes. Because the evaluated programs also differed in vaccination timing, number of

administrations, and vaccine combinations, the independent effect of administration route could not be determined. Among the evaluated protocols, subgroup F2, which included spray administration, showed the highest observed mean HI antibody titer at Day 42 (5.95 ± 0.256). An intermediate observed response was recorded in subgroup D1, which included eye-drop administration, with a mean HI antibody titer of 4.42 ± 0.318 at Day 42. Injection and combined routes showed variable responses across the evaluated protocols. These findings describe differences among complete vaccination protocols and should not be interpreted as evidence of an independent route effect.

Orthogonal comparisons

The original planned-contrast analysis reported differences among selected vaccination protocols. For example, the source analysis reported higher Day-35 titers in A1/A2 than in later-starting schedules (reported $P < 0.0001$), while the reported comparison at Day 42 was not significant ($P = 0.9384$); a comparison of A1/A2 with A4 was reported as $P = 0.0451$. These values are retained here as results from the original analysis, but they should be treated as exploratory rather than confirmatory because each protocol was represented by a single subgroup. The original model output and contrast specifications should be checked before these P values are used to support inferential claims.

The original analysis reported no differences between B1/B2 and B3/B4 at either sampling time ($P > 0.05$), higher Day-42 titers in C1/C2 than in C3/C4 (reported $P = 0.0065$), and higher titers in F1/F2 than in F3/F4 at Day 35 ($P = 0.0009$) and Day 42 ($P = 0.0153$). These reported comparisons are included for transparency but should be interpreted as exploratory, given the lack of independent replication of each vaccination protocol. They do not establish that any single component of a protocol caused the observed differences.

Discussion

The present study describes differences in HI antibody titers among broiler chickens receiving different ND vaccination protocols. Several protocols initiated on Day 1 showed higher observed antibody titers than protocols initiated later. However, because each protocol was represented by a single subgroup and the schedules differed in more than one feature, the observed differences should not be interpreted as confirmatory evidence that early vaccination alone caused a stronger response. Samakkhah et al. [21] and Ahmad et al. [24] have reported associations between vaccination schedules and antibody responses in poultry, providing relevant context for the patterns observed here.

The lower observed titers in some protocols initiated on Day 9 may indicate that later-starting schedules were associated with a different humoral response under the conditions evaluated. Maternal antibodies can influence early vaccine responses, but maternal antibody levels were not measured in the present study; therefore, their contribution to the observed differences cannot be determined directly [25,26].

Differences were observed between protocols involving single ND vaccines and protocols combining ND and AI vaccination. Antigenic competition is one possible explanation discussed in the literature, but it was not measured in this study and should therefore be regarded as a hypothesis rather than a demonstrated mechanism. In addition, the protocols differed in other features, so the observed differences cannot be attributed solely to vaccine formulation. Combined vaccination may nevertheless be useful when protection against multiple diseases is required, although protection was not directly tested here.

An important consideration regarding the external validity of the present findings is the antigenic relationship between the vaccine strains evaluated in this study and the ND virus (NDV) genotypes currently circulating in the region. In particular, genotypes VII.2 and VII.1.1 have been reported in several regions, including the Middle East, and may be associated with outbreaks in vaccinated

flocks. The present study was designed primarily to compare the effectiveness of commonly used ND vaccination components and schedules in commercial broilers; therefore, direct antigenic matching between the vaccine strains (B1, Clone C, and LaSota) and locally circulating field strains was not evaluated. This represents an important area for further investigation, particularly through challenge studies conducted in both commercial and SPF chickens. Nevertheless, a recent challenge study in SPF chickens demonstrated that vaccines mismatched with genotype VII NDV, particularly LaSota and Clone.12IR, were still capable of providing protection against virulent genotype VII NDV and reducing viral shedding (Hassanzadeh et al., 2024). These findings suggest that antigenic mismatch does not necessarily preclude protective efficacy and support the potential relevance of conventional ND vaccine strains in vaccination programs. However, further region-specific challenge studies are required to determine the extent to which antigenic matching influences protection and viral shedding under local epidemiological conditions.

The observed titers varied among protocols that differed in the number and timing of vaccine administrations. Booster vaccination can contribute to antibody responses in poultry, as discussed previously [21], but the present design does not isolate the independent effect of dose frequency. In addition, measurements were limited to Days 35 and 42; the study therefore cannot establish long-term persistence of antibody responses.

The highest observed mean HI titer occurred in subgroup F2, a protocol that included spray administration. However, the F2 schedule also differed from other protocols in timing, number of administrations, and vaccine combinations, so the result cannot be attributed independently to spray delivery. Although spray vaccination can stimulate respiratory mucosal responses, mucosal antibodies such as secretory IgA and local cellular responses were not measured in this study. Accordingly, the present HI data do not demonstrate a mucosal mechanism or establish superior protection by spray vaccination [14,28–30].

Protocols that included injection showed variable systemic HI antibody responses. Any differences from spray- or eye-drop-containing protocols should be interpreted cautiously because route was not isolated from vaccine formulation, timing, and number of administrations. The present study measured serum HI titers and did not directly assess mucosal immunity or clinical protection.

Protocols combining administration routes showed variable HI antibody responses. Because several protocol features differed simultaneously, the present data cannot determine whether combining systemic and mucosal routes improved or reduced the response. Further studies with independent replication and designs that isolate route, timing, and vaccine formulation would be needed to test these questions.

Overall, the observed patterns are broadly relevant to previous reports on vaccination schedules and humoral responses in poultry [32,33]. However, the present findings should be interpreted as descriptive comparisons among the complete protocols tested, not as proof that early vaccination, repeated boosters, or mucosal delivery independently produces stronger or more durable protection. Studies with replicated experimental units, longer follow-up, and challenge or field outcomes would be needed to assess protective efficacy and practical performance.

Conclusion

- Vaccination initiation age: Several protocols initiated on Day 1 were associated with higher observed HI antibody titers. Because protocol features were not independently varied and each protocol lacked independent pen-level replication, this finding should be interpreted descriptively rather than as proof of an independent timing effect.

- Vaccination frequency: HI antibody titers varied among schedules with different numbers of administrations. The present comparisons do not isolate dose frequency as an independent factor and do not establish a general benefit of a five-dose schedule.
- Vaccine formulation: Differences were observed between protocols involving single ND vaccines and combined ND–AI vaccination. These differences cannot be attributed solely to formulation because other protocol characteristics also varied.
- Administration route: The highest observed mean HI antibody titer was recorded in a protocol that included spray administration. The study design does not establish an independent effect of route, and mucosal immunity and clinical protection were not measured.
- In conclusion, this study provides descriptive information on HI antibody titers associated with different ND vaccination protocols in broiler chickens under controlled experimental conditions. The findings should not be interpreted as establishing the causal effect of any single protocol component or the protective superiority of a particular program. Future studies should include independent replication of vaccination protocols and direct measures of protection where feasible.

Materials & Methods

Declaration on AI use

During the preparation of this work, the authors used ChatGPT (OpenAI) for language editing, including grammar correction, improvement of sentence clarity, and stylistic polishing. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Animal rights

The ethical approval for this study was **reported under** grant number 91 **and** Committee on Research Ethics **code 9115005102**, based on the Ethical Guidelines of Research from **the** University of Kurdistan.

Experimental Design, Randomization, and Housing

A total of 1,000 one-day-old specific pathogen-free (SPF) Ross 308 broiler chicks were obtained to evaluate different Newcastle disease (ND) vaccination programs. The chicks were **reported to have been** randomly allocated to six main experimental groups (A–F) and one unvaccinated control group (G). Each main group (A–F) comprised 160 chicks and was divided into four subgroups of 40 chicks each, resulting in 24 vaccination-protocol subgroups (A1–A4, B1–B4, C1–C4, D1–D4, E1–E4, and F1–F4), comprising 960 vaccinated birds in total. Each subgroup received a distinct ND vaccination protocol according to the experimental design. The unvaccinated control group (G) comprised 40 chicks and was housed separately.

Chicks with comparable initial body weights were selected to minimize variability associated with initial body weight. All vaccination-protocol subgroups and the unvaccinated control group were housed in physically separated compartments to prevent direct contact and minimize potential horizontal transmission of live vaccine strains between groups. The control group remained unvaccinated and did not receive any ND vaccine.

Birds were maintained under controlled rearing conditions. Environmental conditions, including temperature, relative humidity, and photoperiod, were monitored and adjusted throughout the study according to the birds' requirements at each growth stage. Feed and water were provided ad libitum. The corn–soybean meal-based diet was formulated according to the Nutrient

Requirements of Poultry [22] to meet the nutritional requirements of each growth stage. Environmental changes were monitored and recorded, and management conditions were kept as consistent as practicable among groups.

The vaccination-protocol subgroup was the level at which the treatment program was assigned. Individual birds within each subgroup served as sampling units for serological measurements and should not be considered independent treatment replicates. The control group consisted of a single separately housed group of 40 birds. If each protocol was indeed represented by only one subgroup, there was no independent pen-level replication for comparing protocols; this design limitation must be considered when interpreting between-protocol differences.

Vaccines

Several commercial Newcastle disease (ND) vaccines and an influenza vaccine were used in this study. The vaccine strains, manufacturers, and routes of administration are summarized in Table 1. The allocation of birds to the experimental groups and subgroups, the vaccination protocols assigned to each subgroup, and the vaccination and serological sampling schedules are summarized in Table 2.

Vaccination programs

ND vaccination protocols were implemented on days 1, 5, 9, 17, and 26 of age, following manufacturer recommendations, standard industry practices, and input from experienced poultry veterinarians. The vaccine types employed included an Inj ND, a combined injectable (Inj ND+AI), and live attenuated ND vaccines comprising B1, Clone C, and LaSota strains. The vaccines were obtained from different manufacturers and were administered according to their respective manufacturers' specifications. Antigenic homology or cross-reactivity between the vaccine strains and specific batches was not independently assessed prior to the trial and was

therefore considered a potential source of variation in the immune responses observed among the vaccination programs.

A total of six experimental groups (A–F) and one unvaccinated control group (G) were established, yielding 25 treatment subgroups in total. Groups A–F each contained four subgroups (e.g., A1–A4), with each subgroup consisting of 40 broiler chicks. The control group (G) consisted of one separate pen containing 40 unvaccinated chicks. Thus, groups A–F contained 960 vaccinated birds in total, while the control group contained 40 birds, resulting in 1,000 birds overall.

The experimental design prioritized four main variables: (i) vaccine type, (ii) age at initial vaccination, (iii) route of administration, and (iv) number of doses administered throughout the rearing period. In Group A, the baseline protocol involved Inj ND, with variations in the timing of the initial dose and subsequent use of live vaccines. Subgroups A1 and A2 received Inj ND on day 1, while A3 and A4 received it on days 5 and 9, respectively. A1 was further vaccinated with B1 (eye drop) on day 5, Clone C (drinking water) on day 9, and LaSota on days 17 and 26. Subgroup A2 received B1 on day 9, Clone C on day 17, and LaSota on day 26. A3 followed a similar schedule, starting with Inj ND on day 5. A4 received Inj ND on day 9, followed by B1 and Clone C on days 17 and 26, respectively.

In Group B, the first dose consisted of a combined Inj ND+AI vaccine. This group was designed to evaluate the effect of co-administering AI antigen with ND vaccine. In Group C, the first dose was a simultaneous administration of Inj ND and B1 via eye drops to assess the immune response elicited by concurrent systemic and mucosal administration. Group D received B1 exclusively via eye drops as the initial vaccine, without any injectable ND vaccine, to evaluate the immune response associated with this administration route. In Group E, both Inj ND and B1 were

administered concurrently at the first vaccination, but B1 was delivered via spray, exploring the immunogenic response associated with simultaneous systemic and spray administration. Finally, Group F was designed to assess the standalone immunogenic response to spray vaccination. Each subgroup (F1–F4) received B1 vaccine via spray at various time points as the initial immunization method. The precise vaccination schedule for each subgroup, including vaccine type, route, and day of administration, is summarized in Table 2.

Blood sampling and serum separation

To evaluate the humoral immune response induced by the vaccination programs, blood samples were collected from the experimental broilers on Days 35 and 42 of the rearing period in accordance with standardized procedures. From each of the 25 experimental subgroups/pens, 10 birds were randomly selected for blood sampling. The same 10 birds from each subgroup/pen were sampled on both Days 35 and 42, resulting in 250 unique birds and a total of 500 blood-sampling observations across the two sampling time points.

Following collection, blood samples were immediately transported to the laboratory under appropriate conditions to minimize the risk of hemolysis, degradation, or contamination. Blood was collected into appropriately labeled tubes and centrifuged at 3000 rpm for 10 min. The resulting serum fraction was carefully separated without disturbing the cellular pellet. Serum aliquots were then stored at -22°C until further serological analysis.

Mortality

Mortality was monitored daily throughout the experimental period. The number of deaths was recorded separately for each vaccination group and the control group (Table 3). Mortality was

expressed as a percentage of the initial number of birds allocated to each group and was calculated as follows.

Mortality (%) = (Number of dead birds / Number of birds initially allocated to the group) × 100.

Immune response evaluation using the HI test

Red blood cell washing

To accurately assess the immune response to vaccination, specifically the antibody levels against NDV, the HI test was employed. A crucial preparatory step in this assay involves the washing of red blood cells (RBCs) to remove potential interfering substances, such as residual plasma proteins.

Initially, anticoagulated whole blood samples were centrifuged to separate plasma from the erythrocytes. Following plasma removal, an equal volume of phosphate-buffered saline (PBS) was added to the RBC pellet, which was then thoroughly resuspended to dilute remaining plasma components. The suspension was centrifuged at 2000 rpm for 10 minutes to pellet the RBCs, and the supernatant was discarded. This washing procedure was repeated three times to ensure maximal removal of plasma proteins and other contaminants. After the final wash, the supernatant appeared clear, confirming the purity of the RBC preparation.

Subsequently, a 1% RBC suspension was prepared by mixing 100 µL of the washed RBCs with 9,900 µL of PBS, yielding a final volume of 10 mL. This standardized suspension was utilized in both the Hemagglutination (HA) and HI assays to evaluate the humoral immune response.

Measurement of antibody titer against NDV

The primary method for quantifying antibody titers was the HI test, which assesses the capacity of serum antibodies to inhibit viral-induced agglutination of red blood cells. The resulting HI titers were used as a measure of the vaccine-induced humoral antibody response.

To perform the HA assay, serial two-fold dilutions of the virus were prepared in a 96-well microplate. Each well initially contained 50 μ L of PBS, into which 50 μ L of the viral stock was added to the first well. Serial dilutions were carried out by transferring 50 μ L from one well to the next through the 11th well, where excess solution was discarded. This dilution series ensured a gradient of viral concentrations.

Then, 50 μ L of the prepared 1% RBC suspension was added to each well, and the plate was incubated for 45 minutes at room temperature to allow viral hemagglutination to occur. The well exhibiting the last visible complete hemagglutination was designated as containing one HA unit. Subsequent wells to the left were assigned HA2 and HA4 units accordingly.

Following HA titration, the HI assay was conducted to detect the presence and concentration of NDV-specific antibodies in serum samples. Fifty microliters of PBS were added to each well, and 50 μ L of serum was introduced into the first well of the microplate. Serial two-fold dilutions of serum were performed up to the 11th well, mirroring the procedure used in the HA assay. Subsequently, 50 μ L of virus containing four HA units (HA4) was added to each well, excluding the negative control wells. The plate was incubated for 30 minutes at 37°C to permit antibody-virus **binding, as confirmed by the authors.**

Following this incubation, 50 μ L of the 1% RBC suspension was added to each well, and the plate was further incubated for 45 minutes **at 37°C**. Wells exhibiting inhibition of hemagglutination—indicated by the absence of RBC clumping—were interpreted as positive for NDV-specific

antibodies at that dilution level. The antibody titer was recorded as the highest serum dilution demonstrating complete inhibition of hemagglutination, expressed as log₂ values. Appropriate positive and negative control sera were included in each assay to ensure validity and reproducibility of results.

Statistical analysis

The hierarchical and repeated-measures structure of the experiment was taken into account in the statistical analysis. Each vaccination-protocol subgroup (A1–A4, B1–B4, C1–C4, D1–D4, E1–E4, and F1–F4) represented a distinct vaccination program and was considered the experimental unit for treatment assignment, whereas individual birds within each subgroup were considered sampling units for serological measurements. Therefore, birds within the same subgroup were not considered independent experimental replicates of the vaccination treatment. Antibody titers were measured in the same individual birds on Days 35 and 42; thus, sampling day was treated as a within-bird repeated factor.

A linear mixed-effects model (LMM) was fitted with vaccination protocol, sampling day, and their interaction as fixed effects and bird identity as a random intercept to account for the correlation between repeated measurements obtained from the same bird. The model was specified as:

$$\text{HI titer} \sim \text{Vaccination protocol} \times \text{Day} + (1 \mid \text{Bird ID})$$

where vaccination protocol represented the assigned vaccination program, Day represented sampling time (35 or 42 days of age), and Bird ID represented the individual bird from which repeated measurements were obtained. Because birds were nested within vaccination-protocol subgroups, the subgroup, rather than the individual bird, represented the experimental unit for treatment assignment. Accordingly, individual birds within a subgroup were not interpreted as

independent treatment replicates. The repeated-measures structure accounted for the correlation between observations obtained from the same bird over time.

Model assumptions were assessed using residual diagnostics. Estimated marginal means (EMMs) were obtained from the fitted LMM using the emmeans framework. Where appropriate, all-pairs comparisons among estimated marginal means were performed with Tukey adjustment for multiple comparisons.

In addition to the all-pairs comparisons, specific contrasts were reported for selected comparisons among vaccination programs presented in Table 4. These contrasts were described in relation to the experimental vaccination schedules, particularly comparisons among programs differing in vaccination timing within the corresponding treatment sets. The exact contrast definitions and multiplicity-adjustment method should be verified against the original R script/output before final submission. Because each protocol appears to have been represented by a single subgroup, the reported P values should not be interpreted as confirmatory evidence of protocol effects.

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Figure legends

Figure 1. Comparative Analysis of HI Titers Based on Vaccination Type and Post-Vaccination Day Across Various Protocols. This figure comprises four-line graphs (A, B, C, D), each illustrating the HI titer on Day 35 (green line) and Day 42 (purple line) post-vaccination. The horizontal axis of each graph categorizes the vaccination type as "Single" or "Dual." The vertical axis consistently represents the HI titer. Each subplot corresponds to a distinct vaccination protocol, indicated by the number of days over which vaccines were administered and the total vaccine count (e.g., "1 DAY - 5 Vaccines" for Graph A, "9 DAY - 3 Vaccines" for Graph D), allowing for a comparison of immune responses under these varying conditions.

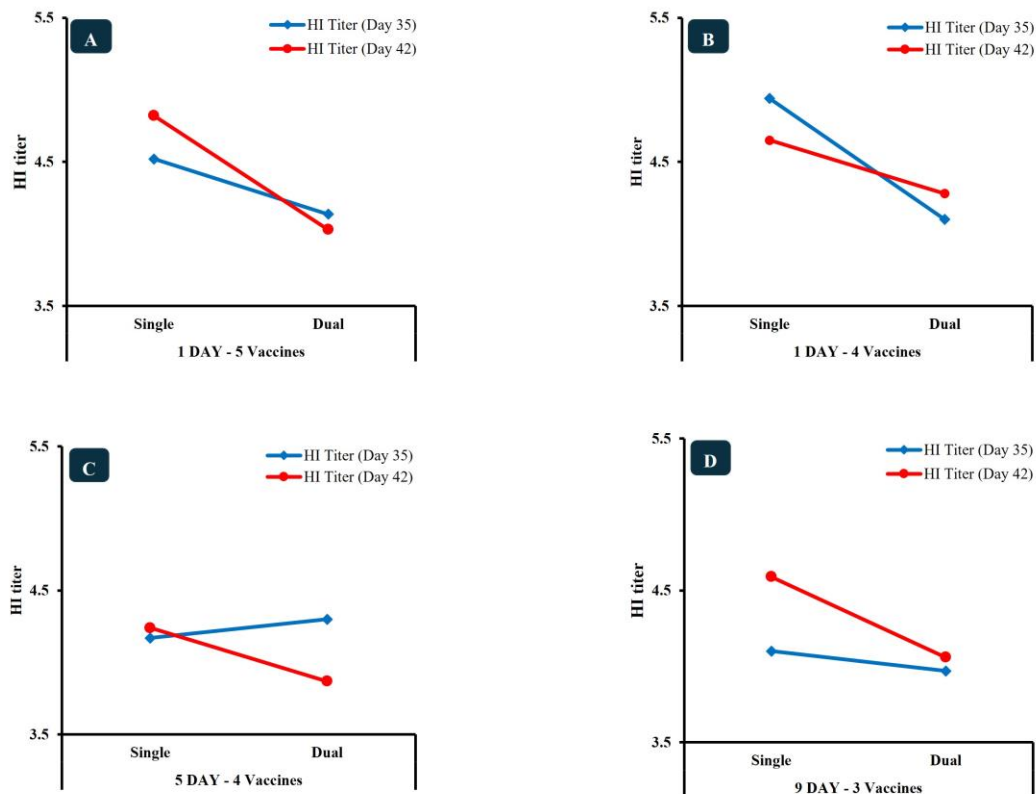


Figure 1.

Figure 2. Comparative analysis of HI titers at day 35 and day 42 post-vaccination across varying vaccination schedules and administration methods in broilers. This figure presents four-line graphs (A, B, C, D), each illustrating the HI titer on Day 35 (green line) and Day 42 (purple line) for different vaccination administration methods. The horizontal axis of each graph specifies the administration method (e.g., Injection, Eye drop, Spray, etc.), while the vertical axis indicates the HI titer. Each subplot corresponds to a unique vaccination protocol, distinguished by the number of days over which vaccines were administered and the total number of vaccines used (e.g., "1 DAY - 5 Vaccines" for Graph A, "9 DAY - 3 Vaccines" for Graph D), allowing for a direct comparison of immune responses under these varied conditions.

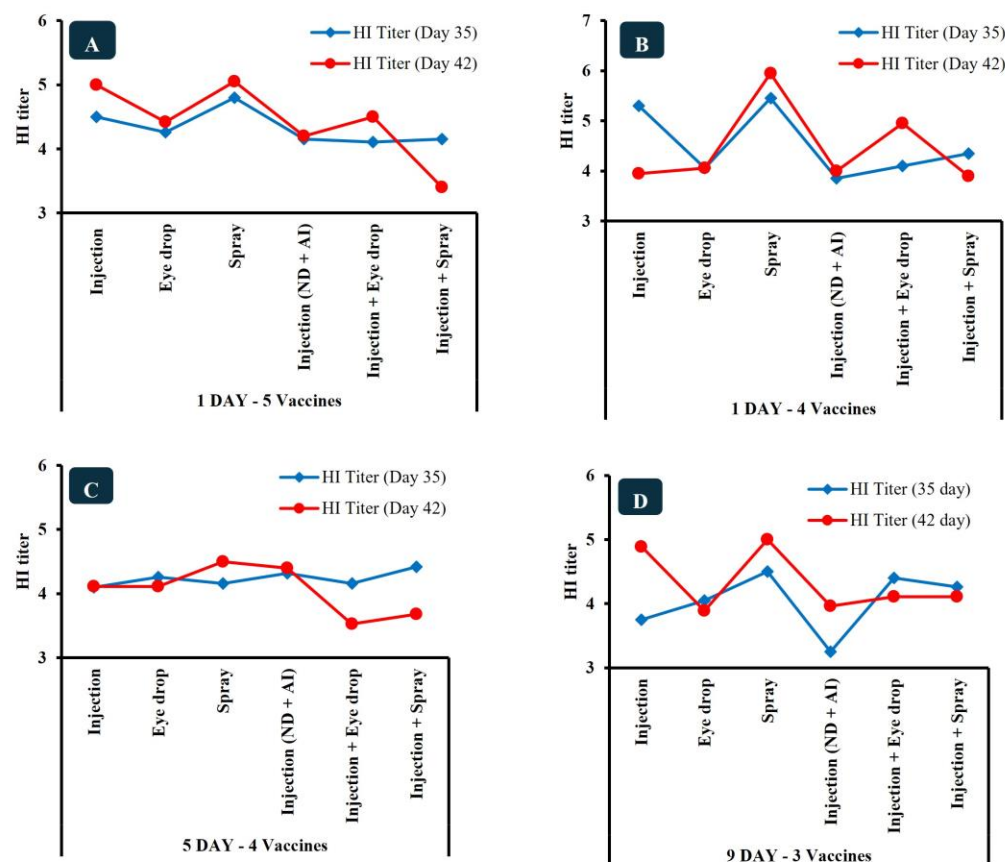


Figure 2.

Figure 3. Comparison of HI titers on Day 35 and Day 42 post-vaccination across different vaccination regimens. This line graph displays two lines representing the HI titer at two different time points: Day 35 (green line) and Day 42 (purple line) post-vaccination.

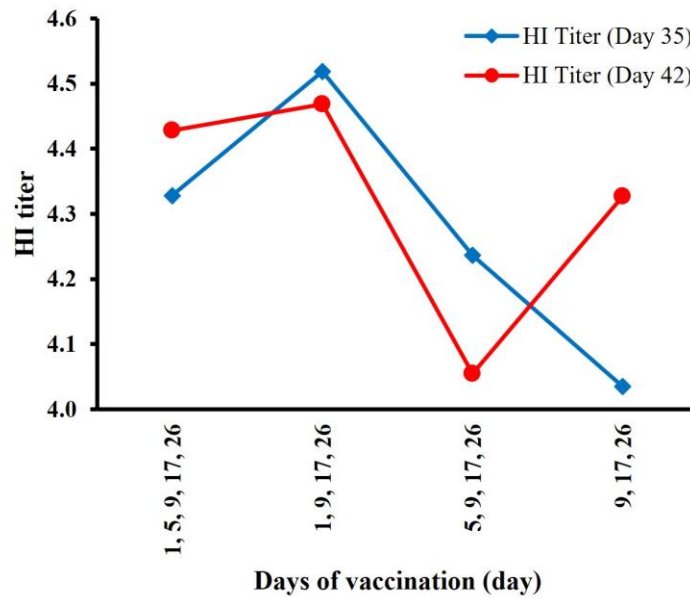


Figure 3.

Table 1. Characteristics and specifications of ND and AI vaccine strains used in the experimental schedules.

Table 2. Simplified summary of ND vaccination protocols in broiler chickens.

Table 3. HI antibody titer levels (Mean $\pm$ SE) against Newcastle Disease Virus at 35 and 42 days of age in experimental groups.

Table 4. Comparison of ND HI antibody titers (Mean $\pm$ SE) at 35 and 42 days of age among main experimental groups.