

Immunogenetic Investigation of the Recombinant Vaccine and its Comparison with Conventional Vaccines in Layer Chickens

Zahraa Bassim Kadhim ^{1*}, Talaat Al-Alwani ¹

¹ Department of Pathology & Poultry diseases, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

ABSTRACT

Background: Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) remain major causes of immunosuppression and mortality in poultry, leading to substantial economic losses in endemic regions. Conventional vaccination often fails due to maternal antibody interference and limited cellular immune stimulation, necessitating innovative vaccine strategies. **Aims:** The purpose of this study was to examine the immunogenic efficacy and mechanism of action of the recombinant HVTND+IBD vaccination against Newcastle Disease (ND) and Infectious Bursal Disease (IBD) in one-day-old layer chicks in comparison to conventional live and killed vaccines. Additionally, it compares the recombinant HVT vaccine with Marek's disease protection induced on by HVT alone. **Methods:** A total of 150 Lohmann Brown chicks were divided into five groups at random: unvaccinated control, live ND+IBD, recombinant HVTND+IBD, HVT alone, and killed ND. ELISA and the DIVA (Differentiation of Infected from Vaccinated Animals) strategy, which differentiation of infected from vaccinated birds based on specific antibody profiles produced by recombinant vaccines, were used to evaluate humoral immune responses. RT-qPCR was used for quantifying viral replication in spleen and bursa tissues. **Results:** RT-qPCR revealed that recombinant HVT replication was slower than HVT alone, and the results demonstrated that the recombinant vaccine induced a slightly delayed but sustained antibody response, peaking at day 21. Around day 10, the recombinant virus was detected. It first replicated in the bursa and spleen before expressing the ND-FP and IBD-VP2 genes. When compared to killed vaccinations, histopathological and immunohistochemical investigations showed significantly higher levels of IL-2 and IL-4 expression in the recombinant and live vaccine groups, indicating enhanced lymphoid activation and stronger cell-mediated immunity. **Conclusion:** These findings support the use of recombinant vaccines for early immunization despite maternal antibody interference, particularly under high pathogen pressure conditions in developing poultry industries.

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1. Introduction

Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) are among the most devastating immunosuppressive agents in poultry, responsible for substantial economic losses due to high mortality rates and compromised flock performance (AL-Zuhariy, 2017; Jumaa et al., 2020). Despite extensive vaccination programs, these pathogens remain endemic in many regions, including Iraq, where viral persistence is frequently reported even in ostensibly well-vaccinated flocks (Hein et al., 2021; Sharif & Ahmad, 2018; Ulaiwi, 2023).

Conventional vaccination protocols—primarily based on live attenuated and inactivated vaccines—remain the mainstay of disease prevention. However, their efficacy is often undermined by several biological and operational constraints, most notably the interference of maternally derived antibodies (MDA) and their limited ability to induce sustained cellular immunity (Birhane & Fesseha, 2020; Mao et al., 2022; Verwoolde et al., 2022). This paradox, wherein vaccinated birds still succumb to infection, highlights the urgent need for novel vaccine platforms that can overcome early-life immunological interference and elicit broader protection. Recombinant vector vaccines, particularly those utilizing herpesvirus of turkey (HVT) as a backbone, have emerged as a promising alternative. These vectors enable simultaneous expression of immunodominant antigens—such as the NDV fusion (F) protein and IBDV VP2—thereby facilitating dual protection in a single administration (Bertzbach et al., 2020; Sun et al., 2024; van Hulten et al., 2021). Additionally, rHVT vaccines exhibit

compatibility with MDA and have demonstrated the ability to activate both humoral and cell-mediated immune pathways (Abou El-Fetouh et al., 2021; Huo et al., 2019).

Nevertheless, there remains limited comparative data evaluating the immunogenic profiles of rHVT-based vaccines against conventional live and killed formulations under controlled early-life vaccination conditions. Most previous studies have focused on single endpoints—seroconversion, viral shedding, or histopathology—without integrating molecular, cellular, and histological analyses in a unified experimental framework, particularly in the context of Iraqi poultry production.

Therefore, this study was designed to systematically compare the immunological, molecular, and histopathological effects of a recombinant HVT-ND+IBD vaccine with those of conventional live and killed ND/IBD vaccines in one-day-old layer chicks. By integrating serological (ELISA and DIVA), molecular (RT-qPCR), and tissue-level (histopathology) assessments, this research aims to elucidate the relative efficacy of each vaccine strategy, particularly during the critical window of maternal antibody interference. The findings are expected to provide valuable guidance for optimizing poultry vaccination protocols under endemic and biosecurity-challenged field conditions.

2. Materials and Methods

2.1 Ethical Approval

All procedures involving animals were approved by the Committee for Animal Use and Care, College of Veterinary Medicine, University of Baghdad (Approval No. 2360; issued on December 12, 2024), and conducted in accordance with institutional and national ethical guidelines for animal research.

2.2 Experimental Design and Animal Management

A total of 150 one-day-old Lohmann Brown layer chicks, obtained from a commercial hatchery, were randomly assigned into five experimental groups ($n = 30$ per group). All birds were unvaccinated at baseline and were housed in separate, disinfected compartments under controlled environmental conditions. Disinfection was carried out using sodium hypochlorite followed by potassium permanganate fumigation. After a 48-hour aeration period, chicks were placed on clean cardboard-lined wood shavings and provided ad libitum access to commercial feed and water.

The brooding temperature was initially maintained between 30–28 °C during the first week and gradually reduced to 25–26 °C by day 35. Strict biosecurity measures were applied to prevent cross-contamination between groups.

2.3 Vaccination Protocol

- Group 1 (G1): Vaccinated subcutaneously at day 1 with recombinant HVT-ND+IBD vaccine.
- Group 2 (G2): Vaccinated subcutaneously at day 1 with HVT vaccine only.
- Group 3 (G3): Received live ND vaccine via eye drop on days 1 and 14, and live IBD vaccine subcutaneously at day 1.
- Group 4 (G4): Received inactivated (killed) ND vaccine subcutaneously at day 1.
- Group 5 (G5): Served as unvaccinated negative control. (See Figure 1).

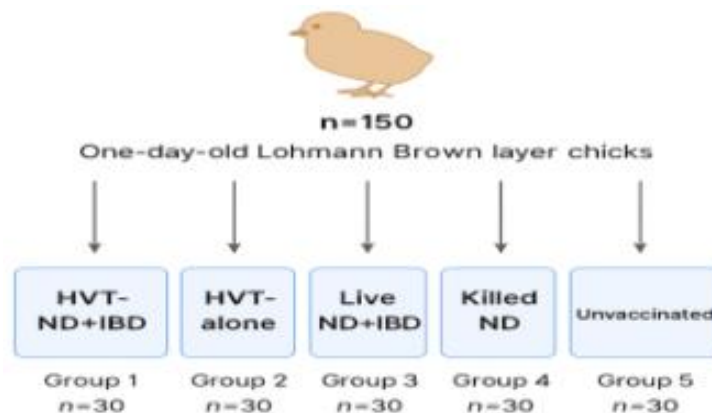


Figure 1: Experimental Design

2.4 Sample Collection

Blood samples (1–2 mL) were collected from the jugular vein of five birds per group on days 1 (baseline), 10, 21, and 35 post-vaccination. Sera were separated by centrifugation at 2500 rpm for 15 minutes and stored at –20 °C until

serological testing.

For molecular and histopathological analyses, spleen and bursa of Fabricius samples were collected from:

- 5 birds per group on days 10 and 21,
- 10 birds per group on day 35.

Tissue samples were divided: one half was snap-frozen in liquid nitrogen and stored at -80°C for DNA extraction; the other half was fixed in 10% neutral-buffered formalin for histopathology.

2.5 Serological Evaluation (ELISA and DIVA)

Antibody responses to NDV and IBDV were quantified using commercial ELISA kits (ID.vet, France) according to the manufacturer's instructions (Al-Zuhariy, 2023). Each sample was run in triplicate. The Differentiation of Infected from Vaccinated Animals (DIVA) test was used to detect antibodies specific to NDV fusion protein (F) and IBDV VP2 protein.

2.6 Viral DNA Extraction and Quantification (RT-qPCR)

DNA was extracted from bursa and spleen tissues using the Gene-spin™ Viral DNA/RNA Extraction Kit (Intron, Korea). Quantitative real-time PCR was conducted using GoTaq® Probe qPCR Master Mix (Promega, USA) targeting the *sorf1* gene of HVT (MeHV-1), with specific primers and a FAM-labeled probe as described by Salah et al. (2020) and Hameed et al. (2022).

PCR cycling was performed using a Stratagene thermal cycler with the following conditions:

- Initial denaturation: 95°C for 10 minutes;
- 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Absolute quantification was achieved using standard plasmid DNA serial dilutions (10^7 to 10^1 copies) to generate a standard curve. The regression equation was used to calculate viral copy number:

$$\text{Copy Number} = 10^{(\text{Ct} - b) / m}$$

Only reactions with $R^2 > 0.98$ were included. All reactions were run in triplicate. Negative samples were reported as "No Ct".

2.7 Histopathological Examination

Formalin-fixed tissues were processed using standard paraffin embedding. Sections of 4–6 μm thickness were cut and stained with hematoxylin and eosin (H&E). Slides were examined under light microscopy at 100 $\times$ and 400 $\times$ magnifications to assess follicular integrity, cortical-medullary structure, and lymphoid activation in both spleen and bursa.

2.8 Immunohistochemical analysis

Immunohistochemical analysis was performed using anti-IL-2 and anti-IL-4 primary antibodies to detect cytokine expression in spleen and bursa samples. The staining intensity and localization were examined at 100 $\times$ and 400 $\times$ magnifications and quantified using QuPath software. This aimed to evaluate the extent of T and B cell activation among the different vaccinated groups.

2.9 Statistical Analysis

Normality and homogeneity of variances were verified using the Shapiro–Wilk and Levene's tests, respectively. Data meeting parametric assumptions were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Significance was set at $p \leq 0.05$. Statistical analyses were performed using IBM SPSS v26 and GraphPad Prism v9.5.

3. Results

All quantitative data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine significant differences between experimental groups. In the maternal immunity comparisons (Tables 1 and 2),

Table 1: Maternal NDV IgY Titers (Mean $\pm$ SE) at Day 1 in Chicks from Breeders Vaccinated with Conventional vs. Recombinant Vaccines

Vaccine Type	NDV Titer (Mean $\pm$ SE)	Significance
Conventional	17,798 $\pm$ 1070	* $p \leq 0.05$
Recombinant (DIVA)	12,830 $\pm$ 55	* $p \leq 0.05$

Note: Titers Measured by ELISA. Statistical Comparison Performed Using Independent t-test

Table 2: Maternal IBDV IgY Titers (Mean $\pm$ SE) at Day 1 in Chicks from Breeders Vaccinated with Conventional vs. Recombinant Vaccines

Vaccine Type	IBDV Titer (Mean $\pm$ SE)	Significance
Conventional	4862 $\pm$ 481	* $p \leq 0.05$
Recombinant (DIVA)	11,510 $\pm$ 759	* $p \leq 0.05$

Note: Titers Measured by ELISA. Statistical Comparison Performed Using Independent t-Test

Paired t-tests revealed significant differences ($p \leq 0.05$) between conventional ELISA and DIVA in measuring day-one NDV and IBDV antibodies, suggesting higher sensitivity of the DIVA assay for IBDV. For NDV and IBDV humoral responses (Tables 3 and 4).

Table 3: NDV Antibody Titers (Mean $\pm$ SE) at Days 10, 21, and 35 Post-Vaccination in Layer Chicks

Group	Day 10	Day 21	Day 35
Recombinant (G1)	1541 $\pm$ 108 ^c	12,172 $\pm$ 74 ^a	9843 $\pm$ 235 ^a
Live ND+IBD (G3)	20,720 $\pm$ 54 ^a	11,880 $\pm$ 2725 ^a	646 $\pm$ 86 ^b
Killed ND (G4)	18,021 $\pm$ 650 ^a	5406 $\pm$ 1607 ^b	8278 $\pm$ 2079 ^a
Control (G5)	13,320 $\pm$ 1232 ^b	6139 $\pm$ 1439 ^b	284 $\pm$ 10 ^b

Different superscript letters indicate significant differences (Tukey's test, $p \leq 0.05$ / 0.01) LSD: 2245.9 at Day 10, 5212.3 at Day 21, 3143.0 at Day 35.

Different superscript letters (a, b, c) indicate significant differences between groups (Tukey's HSD, $p \leq 0.05$)

Table 4: IBDV Antibody Titers (Mean $\pm$ SE) at Days 10, 21, and 35 Post-Vaccination in Layer Chicks

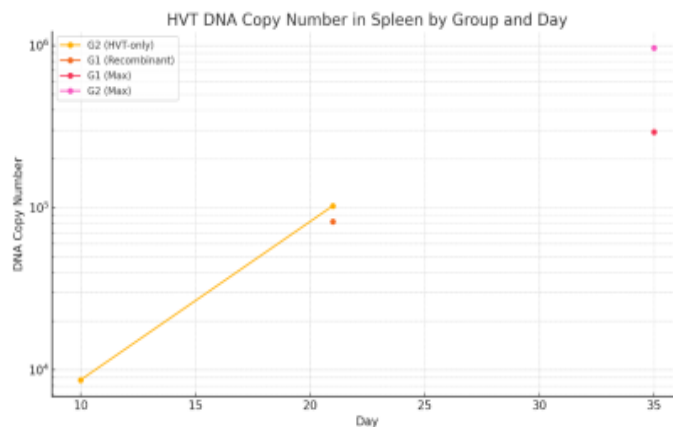
Group	Day 10	Day 21	Day 35
Recombinant (G1)	8587 $\pm$ 1063 ^a	1809 $\pm$ 496 ^a	2492 $\pm$ 1057 ^a
Live ND+IBD (G3)	4370 $\pm$ 975 ^b	1044 $\pm$ 504 ^{ab}	151 $\pm$ 82 ^b
Control (G5)	1256 $\pm$ 252 ^c	273 $\pm$ 73 ^b	260 $\pm$ 115 ^b

LSD: 2605.8 at Day 10, 1265.8 at Day 21, 1898.4 at Day 35.

Different superscript letters (a, b, c) indicate significant differences between groups (Tukey's HSD, $p \leq 0.05$).

Intergroup comparisons across time points (10, 21, and 35 days) showed statistically significant variations in antibody titers ($p < 0.01$ or $p < 0.05$). The recombinant vaccine (G1) demonstrated delayed but sustained responses, particularly evident at 21 and 35 days, while the live vaccine group (G3) showed rapid but transient immunity. Different superscript letters in the same column indicate statistically significant differences (Tukey's test, $p \leq 0.05/0.01$), with least significant differences (LSD) specified for each timepoint.

Intergroup comparisons of viral DNA copy number in spleen tissues revealed markedly higher replication kinetics in the HVT-only group (G2) across all timepoints, with peak values observed on day 35. The recombinant group (G1) displayed a delayed yet significant increase in HVT copy numbers from day 21 onwards, consistent with the known replication lag associated with rHVT constructs. No Ct values on day 10 in G1 reflect absent or minimal early replication (Figure 2).

**Figure 2:** Quantification of HVT DNA Copy Numbers in the Spleen by RT-qPCR across Days 10, 21, and 35 Post-Vaccination

The viral load is expressed as log₁₀-transformed copy number. Group G1 (Recombinant HVT-ND+IBD) showed no detectable viral replication at Day 10 ("No Ct"), followed by a delayed increase at Day 21 and heterogeneously elevated

levels at Day 35. Group G2 (HVT-only) showed earlier and more consistent replication patterns across all timepoints. Data were derived from triplicate reactions. Error bars were omitted due to heterogeneous distribution.

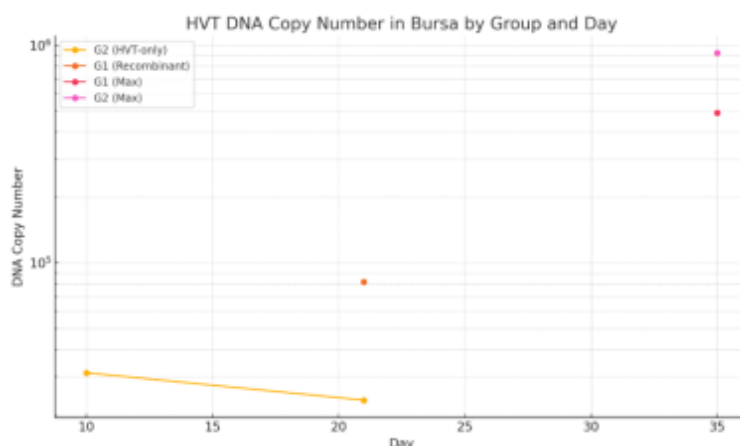


Figure 3: HVT DNA Copy Numbers in the Bursa of Fabricius by RT-qPCR at Days 10, 21, and 35 Post-Vaccination

Viral load was assessed using SYBR Green-based RT-qPCR and standard plasmid curves. Group G1 (Recombinant) exhibited delayed onset of replication (undetectable at Day 10), peaking at Day 35. Group G2 (HVT-only) displayed earlier replication with peak values at Day 35. Data expressed as log₁₀-transformed values (Figure 3).

Histopathological examination of the bursa of Fabricius and spleen revealed progressive alterations among the experimental groups. In the control negative group (Figure 4), the lymphoid follicles exhibited a well-defined cortical and medullary organization, with the cortex displaying darker staining and higher cellularity compared to the paler, less cellular medulla. The center of the follicle showed low cellular density, indicating a quiescent follicular status. Similar architectural features were observed in the HVT group (Figure 5), although some follicles demonstrated increased central cellularity, suggesting mild immune activation.

In the Killed ND group (Figure 6), there was a notable increase in central follicular cellularity compared to the control, indicative of enhanced lymphoid activity. The Live ND+IBD group (Figure 7) showed pronounced lymphocytic hyperplasia, resulting in the loss of clear corticomedullary distinction in several follicles. Some follicles appeared inactive, characterized by low central cellular density. The most severe histopathological changes were observed in the Recombinant ND+IBD group (Figure 8), where marked lymphoid hyperplasia and obliteration of follicular architecture were present in most tissue sections, denoting a strong immunological response.

In splenic tissues, the control negative group (Figure 9) exhibited normal architecture, with the white pulp comprising less than 50% of the splenic area. In contrast, the HVT (Figure 10) and Killed ND (Figure 11) groups showed expansion of the white pulp covering approximately 50% of the area. This proportion increased to more than 60% in the Live ND+IBD group (Figure 12) and exceeded 70% in the Recombinant ND+IBD group (Figure 13), reflecting progressive immunostimulation associated with the different vaccine strategies.

3.1 Histopathological Results

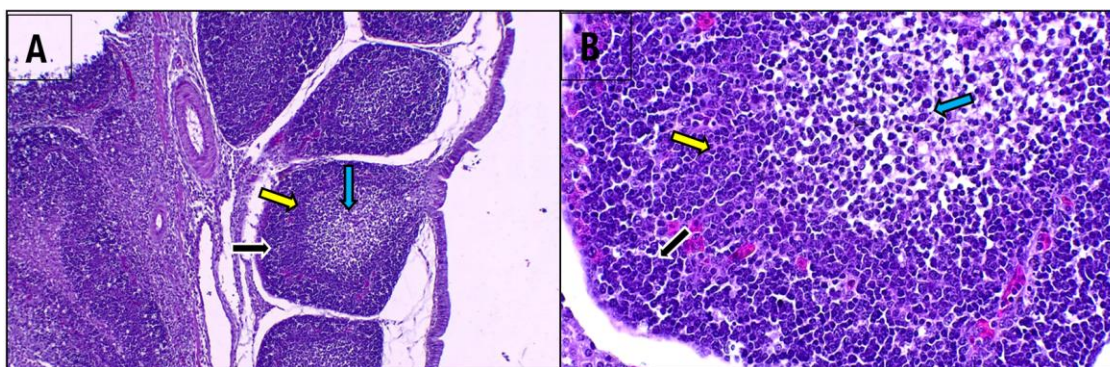


Figure 4: Photomicrograph of the Bursa Fabricius of Control Negative Group. A&B/ Note the Lymphoid Follicles Showed Notable Cortex (Black Arrow) and Medulla (Yellow Arrow), Where the Cortex Cells Showed Darker Color Compared with Medulla That Showed Pale Color. Also, The Center of Follicle Showed Low Cellular Density (Blue Arrow). However, These Features Were Observed in Most Tissue Areas. H&E. A: 100x and B; 400x.

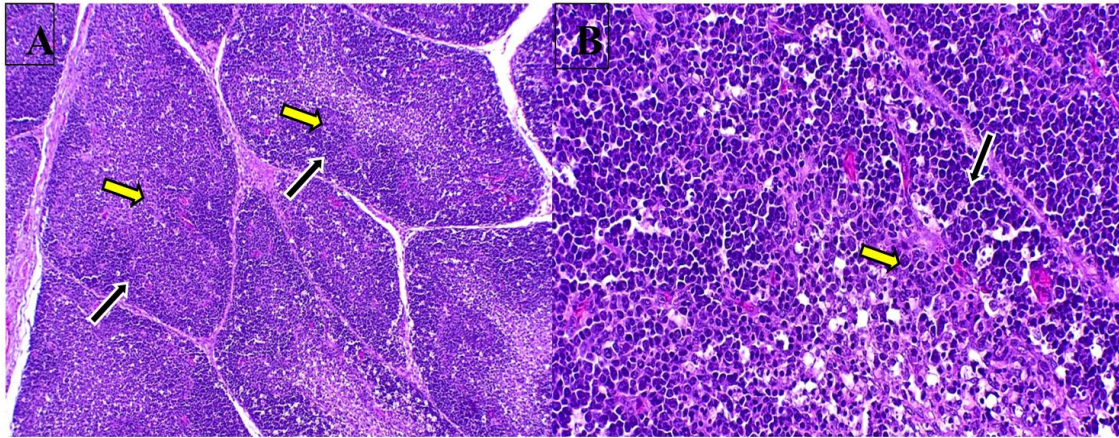


Figure 5: Photomicrograph of the Bursa Fabricius of HVT Group. A& B/ Note the lymphoid Follicles Showed Notable Cortex (Black Arrow) and Medulla (Yellow Arrow), Where the Cortex Cells Showed Darker Color Compared with Medulla that Showed Pale Color. Also, the Center of Follicle Showed Low Cellular Density (Blue Arrow), However Some Follicles Shoed Higher Cellular Density in the Affected Bursa Fabricius. These Features Were Observed in most Tissue Areas. H&E. A: 100x and B; 400x.

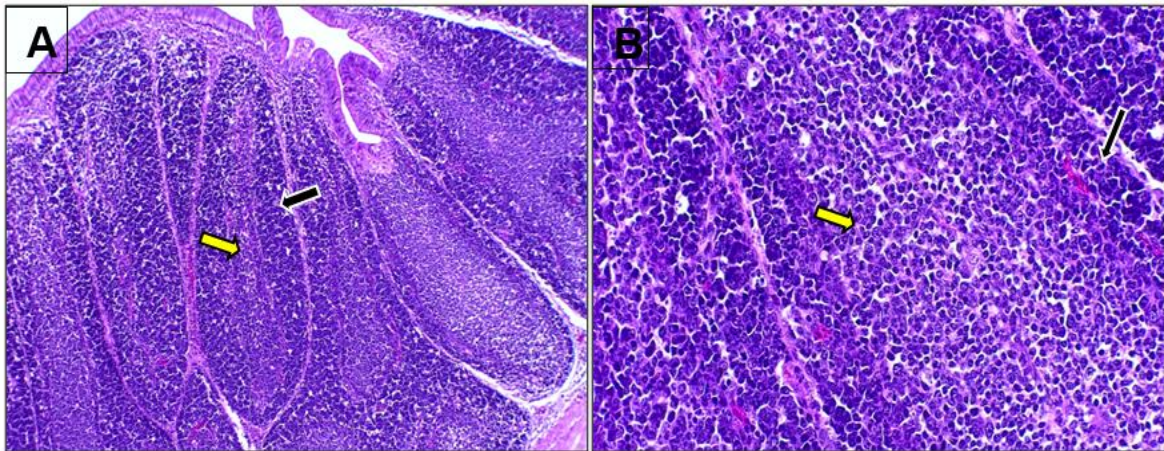


Figure 6: Photomicrograph of the Bursa Fabricius of K. ND Group. A& B/ Note the lymphoid Follicles Showed Notable Cortex (Black Arrow) and Medulla (Yellow Arrow), Where the cortex cells showed darker Color Compared with Medulla that Showed Pale Color. However, the Center of Follicle Showed Higher Cellular Density (Blue Arrow) Compared with Control Negative Group. However, these Features were Observed in most Tissue Areas. H&E. A: 100x and B; 400x.

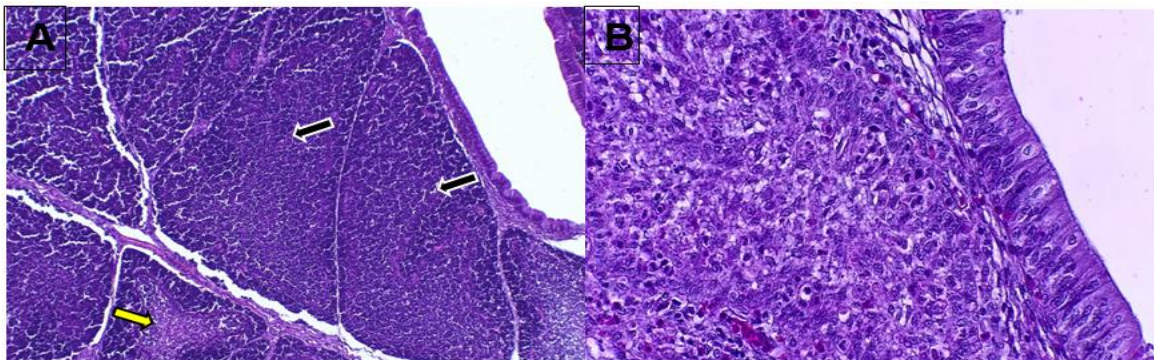


Figure 7: Photomicrograph of the Bursa Fabricius of L. ND+ IBD Group. A& B/ Note the Cortex and Medulla Areas are Difficult to Recognize Due to Lymphocytes Hyperplasia in Medulla Areas. However, Some Follicles are not Completely Active, Where the Center of Follicle Showed Low Cellular Density (Yellow Arrow) Compared with Active Follicles. H&E. A: 100x and B; 400x.

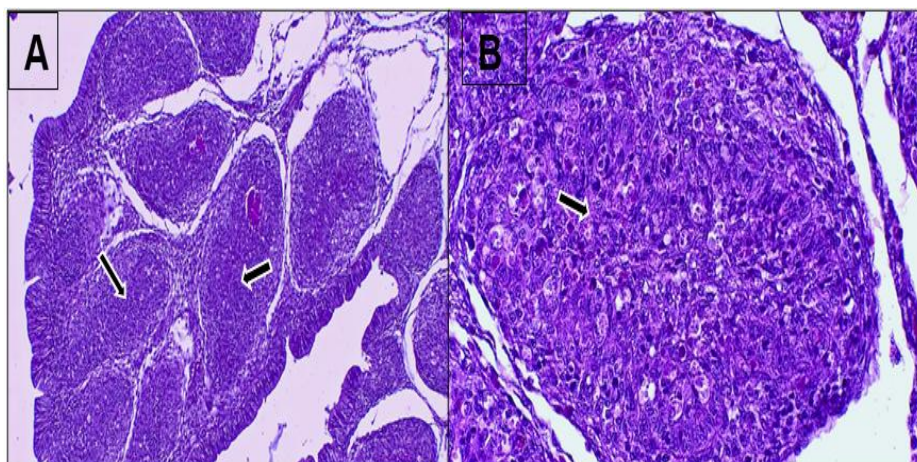


Figure 8: Photomicrograph of the Bursa Fabricius of R. ND+ IBD group. A& B/ Note the Cortex, and Medulla Areas are Difficult to Recognize Due to Lymphocytes Hyperplasia (Black Arrow) in Medulla Areas, Indicating the Active Follicles. However, These Features were Observed in Most Areas of the Bursa Fabricius. H&E. A: 100x and B; 400x.

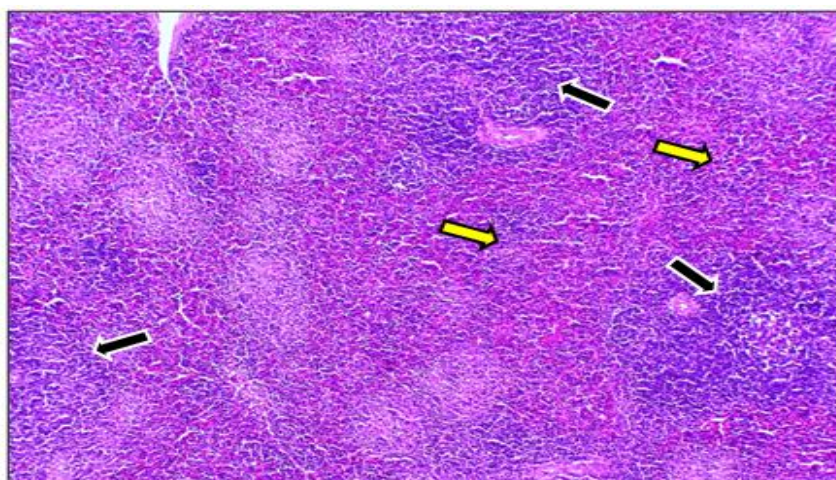


Figure 9: Photomicrograph of the Spleen of Control Negative Group. Normal Histological Architecture of Spleen, However the White Pulp Region (Black Arrow) Covers Less than 50% of splenic Area Compared with Red Pulp Region (Yellow Arrow). H&E. 100x.

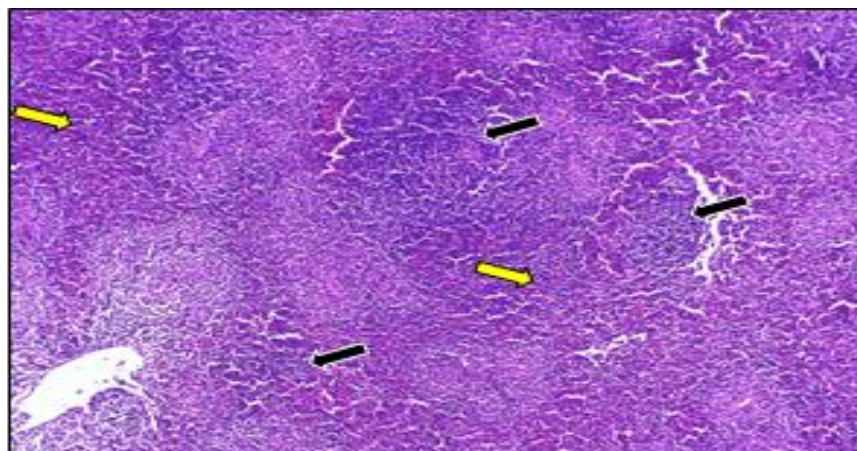


Figure 10: Photomicrograph of the Spleen of HVT Group. Normal Histological Architecture of Spleen, However the White Pulp Region (Black Arrow) Covers about 50% of splenic area Compared with Red Pulp Region (Yellow Arrow). H&E. 100x.

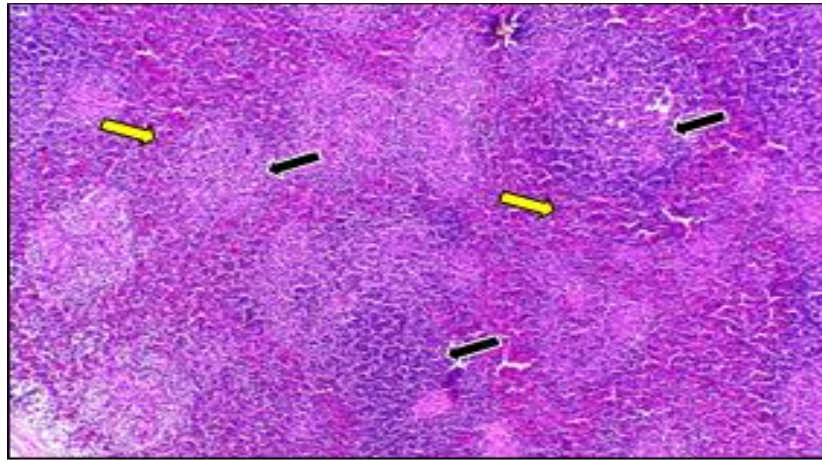


Figure 11: Photomicrograph of the Spleen of K. ND Group. Note the White Pulp Region (Black Arrow) Covers about 50% of Splenic Area Compared with Red Pulp Region (Yellow Arrow). H&E. 100x.

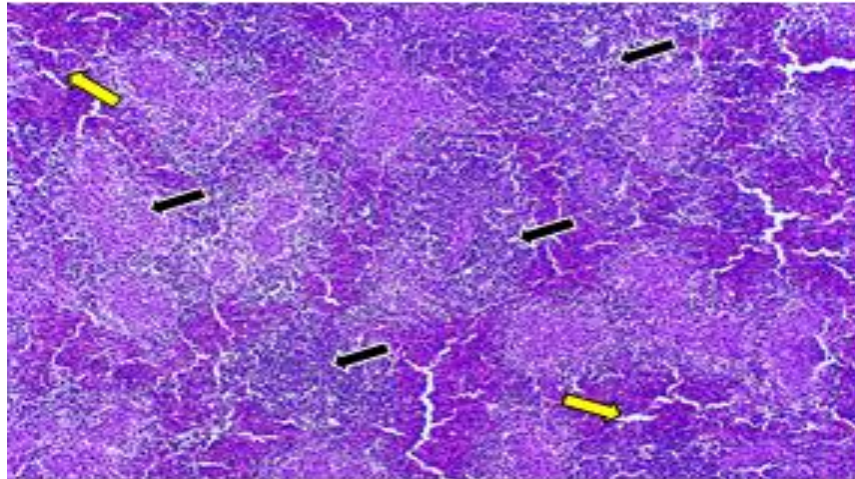


Figure 12: Photomicrograph of the Spleen of L. ND+ IBD group. Note the White Pulp region (Black Arrow) Covers More than 60% of Splenic Area Compared with Red Pulp Region (Yellow Arrow). H&E. 100x.

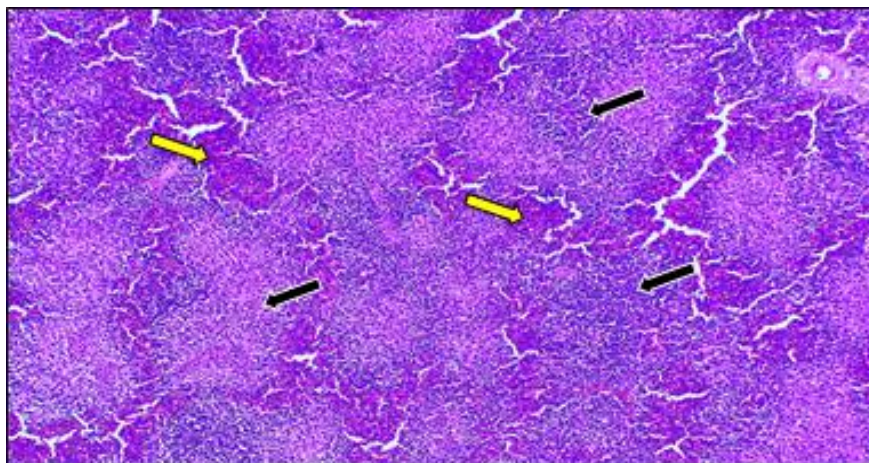


Figure 13: Photomicrograph of the Spleen of R. ND+ IBD Group. Note the White Pulp Region (Black Arrow) Covers More than 70% of Splenic Area Compared with Red Pulp Region (Yellow Arrow). H&E. 100x.

3.2 Immunohistochemistry Results

Due to the high number of IL-2 immunohistochemical photomicrographs and their overlapping descriptive features,

a comparative summary table was constructed to synthesize the observed patterns. Original images are available upon request.

The summarized tables provide a comparative overview of IL-2 and IL-4 immunohistochemical expression patterns across the bursa of Fabricius and spleen in various experimental groups. Both cytokines exhibited minimal expression in control groups, with gradually increasing intensity from killed (K. ND) to live (L. ND+IBD) and recombinant (R. ND+IBD) vaccine groups. Notably, the recombinant vaccine group demonstrated the strongest and most widespread expression of both IL-2 and IL-4, particularly in cortical, medullary, and intra-follicular regions of the bursa, as well as in the white and red pulp zones of the spleen. These findings suggest enhanced cell-mediated immune activation and lymphoid stimulation, consistent with the immunogenic profile expected from recombinant vector vaccines.

Table 5: Comparative IL-2 Expression in Bursa and Spleen among Experimental Groups

Group (Code)	Tissue	IL-2 Expression	Location of Expression	Notable Observations
Control Negative	Bursa	Weak	Few lymphocytes in cortex (black arrow)	Expression limited to cortex; low cellular density in follicle center
	Spleen	Weak	Center of white pulp follicles (black arrow)	No expression in red pulp; patchy distribution
K. ND	Bursa	Weak	Cortex and intra-follicular regions (yellow arrow)	T-cell aggregation noted; increased cortical activity
	Spleen	Moderate	Lymphatic follicle and PALS; red pulp (yellow arrow)	Discrete expression in both pulp regions
L. ND+IBD	Bursa	Moderate	Cortex and medulla; intra-follicular (yellow arrow)	T-cell aggregation and bilateral follicular activation
	Spleen	Moderate to Strong	Follicle center and red pulp	Strong expression in red pulp, but not throughout
R. ND+IBD	Bursa	Strong	Cortex, medulla, and intra-follicular (yellow arrow)	Dense IL-2 expression, active follicles across tissue
	Spleen	Strong	Entire white pulp and red pulp regions	High-density expression in all lymphatic areas

IL-2 protein expression was assessed via DAB immunohistochemical staining at 100x and 400x magnifications. Expression intensity was categorized based on density and tissue distribution, as inferred from microscopic observations.

Table 6: Summary of Interleukin-4 (IL-4) Immunohistochemical Expression in Bursa and Spleen Tissues across Different Vaccinated Chick Groups

Group (Code)	Tissue	IL-4 Expression Intensity	Expression Location	Key Histological Findings
Control Negative	Bursa	Weak	Cortex of lymphoid follicles only	No IL-4 expression in intra-follicular areas
	Spleen	Weak	Centers of white pulp follicles	Absent in red pulp regions
K. ND	Bursa	Weak to Moderate	Cortex and medulla	Stronger than control; intra-follicular expression absent
	Spleen	Weak	White pulp center; limited in red pulp (yellow arrow)	Expression sparse and restricted to small areas
L. ND+IBD	Bursa	Moderate	Cortex and medulla	Higher than K. ND and control; no intra-follicular staining
	Spleen	Strong	Follicles and PALS in white pulp; ~50% red pulp (yellow arrow)	Marked increase in red pulp expression
R. ND+IBD	Bursa	Strong	Cortex and medulla	Dense expression throughout follicular compartments
	Spleen	Strong	Follicles, PALS, and most of red pulp	Nearly complete red pulp involvement

Expression levels were assessed via DAB-based IHC staining and categorized based on intensity and anatomical localization. Expression in intra-follicular and red pulp regions is highlighted for immunological interpretation.

4. Discussion

The present study provides a comprehensive evaluation of the immunological, molecular, and histopathological responses elicited by recombinant and conventional ND+IBD vaccines in layer chicks. The findings reveal that the recombinant HVT-ND+IBD vaccine, although characterized by a delayed onset of activity, ultimately induces a more durable and integrated immune response than both live and inactivated vaccine counterparts (Hameed et al., 2022).

The maternal antibody titers recorded at day one underscore the influence of vaccine type administered to breeder hens. NDV-specific IgY levels were higher in progeny from conventionally vaccinated breeders, whereas IBDV titers were elevated in those from rHVT-VP2-vaccinated hens (Tables 1 and 2). This discrepancy aligns with reports by Oviedo-Rondon

et al. (2013) and Alizadeh et al. (2017), who emphasized the dependence of maternal antibody transfer on both the antigen load and timing of vaccination in breeder flocks (Terfa et al., 2015).

The antibody kinetics observed post-vaccination further distinguish the immunological profiles of each vaccine group. Live vaccines (G3) triggered an early surge in NDV-specific antibodies by day 10, which then declined substantially by day 35 (Table 3). Conversely, the recombinant group (G1) exhibited a delayed yet sustained humoral response that peaked at day 21 and persisted through day 35. These results are in agreement with OMRAN (2018), Hu et al. (2022), and Edan and Jumaa (2025), who reported that rHVT-based vaccines replicate more slowly but ensure prolonged antigen exposure. The slower immune priming is attributed to the intracellular expression of the NDV-F and IBDV-VP2 antigens within infected host cells, resulting in efficient stimulation of both MHC-I and MHC-II pathways.

RT-qPCR data further validated these trends, as viral DNA was undetectable in the recombinant group (G1) at day 10 but showed substantial replication by day 21 and peaked heterogeneously at day 35 (Figure 2). These results confirm the delayed replication kinetics of the recombinant virus compared to HVT-alone (G2), which displayed earlier and more uniform tissue replication (Haque et al., 2024). The delayed peak in rHVT replication likely reflects constraints related to foreign gene insertion, as previously noted by Gelb Jr et al. (2016) and Bull et al. (2019), who reported reduced genome stability and expression efficiency in multi-antigen recombinant constructs.

The occurrence of “No Ct” values in several samples, particularly in the recombinant vaccine group (G1) on day 10 and partially on day 35, is not indicative of technical error but rather reflects biological heterogeneity in viral replication kinetics. Such variability may arise from differences in cellular tropism, immune-mediated clearance of the vector, or epigenetic silencing of recombinant viral promoters. Previous studies, such as those by Wang et al. (2021) and Islam et al. (2013), suggest that host immune regulation and chromatin remodeling at viral insertion sites may suppress early transcription, especially in multi-antigen constructs like rHVT-ND+IBD.

Histologically, the recombinant vaccine group demonstrated the most pronounced lymphoid activation. In the bursa of Fabricius, lymphoid follicles lacked distinct corticomedullary zoning due to intense lymphocytic hyperplasia (Figure 8). The spleen displayed a white pulp area exceeding 70% of total splenic tissue (Figure 13), compared to ~50% in G2 and G4 (Figures 10–11) and <50% in controls (Figure 9). These findings reflect increased lymphopoiesis and germinal center development, in agreement with Gewaily et al. (2023), Khalaf and Ali (2023), and Wegner et al. (2025).

The spleen, being a critical lymphoid organ, undergoes substantial changes in architecture following immunization. The expansion of white pulp, which contains B and T lymphocytes, indicates heightened immune activation. According to Chung et al. (2021) and Q. Qiao et al. (2021), the proliferation of germinal centers within the white pulp is a hallmark of active antigen presentation and humoral response initiation. These histological findings further support the enhanced immunogenicity of rHVT vaccines.

The immunohistochemical findings summarized in Tables X and Y provide further evidence supporting the superior immunostimulatory capacity of the recombinant HVT-ND+IBD vaccine. Notably, both IL-2 and IL-4—critical cytokines mediating T-cell activation and B-cell proliferation—showed a gradual increase in expression intensity from the killed vaccine group (K. ND), through the live vaccine group (L. ND+IBD), to the recombinant vaccine group (R. ND+IBD). This pattern was evident across both primary lymphoid tissue (bursa of Fabricius) and secondary lymphoid tissue (spleen), with the recombinant group displaying the most robust and spatially extensive staining, particularly in intra-follicular and periarterial lymphatic sheath regions.

These findings align with previous studies demonstrating that recombinant vector vaccines elicit stronger Th1- and Th2-type immune responses due to prolonged antigen presentation and enhanced cellular immunity (Costa & Sautto, 2024; N. Qiao et al., 2021). IL-2, a hallmark of T-cell proliferation, and IL-4, a key driver of B-cell class switching, serve as reliable indicators of vaccine-induced lymphoid activation. The broader distribution and higher intensity of cytokine expression in the recombinant group reflect the heightened functional activity of immune compartments, which is crucial for effective viral clearance and long-term protection (Balachandran, 2022; Ritzau-Jost & Hutloff, 2021). Moreover, the parallel increase in both cytokines within the red pulp of the spleen indicates not only follicular activation but also enhanced systemic immune mobilization, supporting the hypothesis that recombinant vaccines can overcome maternal antibody interference and induce potent cell-mediated immunity (Brennan, 2021; Cinicola et al., 2021). Therefore, the histological patterns observed provide histopathological validation of the quantitative and serological findings discussed earlier, reinforcing the immunological advantages of rHVT-based vaccination platforms under endemic field conditions.

5. Conclusion

The recombinant HVT-ND+IBD vaccine induced a delayed but sustained immune response, with superior antibody titers and lymphoid activation compared to live and inactivated vaccines. Its ability to overcome maternal antibody interference and promote balanced immunity highlights its suitability for early vaccination. These findings support

integrating rHVT-based vaccines into poultry immunization programs, particularly in endemic settings with high exposure pressure. Moreover, the robust immunohistochemical expression of IL-2 and IL-4 observed in the recombinant vaccine group reinforces its ability to stimulate both T-helper 1 and T-helper 2 immune pathways, which are essential for long-lasting and effective immunity in early-vaccinated chicks.

6. Limitations

This study was limited by a relatively short observation window and the absence of long-term immunological tracking, particularly regarding memory T-cell activation and protective efficacy under field exposure conditions. Additionally, RT-qPCR comparisons were presented descriptively without inferential statistical analysis, and histological assessments were semi-qualitative in nature.

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