UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY OF NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN COMMERCIAL BROILER CHICKENS

CHONG HAO HAN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Pathogenicity of Novel Variant Infectious Bursal Disease Virus in Commercial Broiler Chickens”, by Chong Hao Han and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

PROFESSOR DATO’ DR MOHD BIN HAIR BEJO
DVM (UPM), PhD (LIVERPOOL)
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

DR MAZLINA BINTI MAZLAN
DVM (UPM), MS (UPM), PhD (UPM)
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

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LIST OF ABBREVIATIONS

BBIX	Bursa body weight index
BF	Bursa of Fabricius
B/BW	Bursa-to-body-weight ratio
caIBDV	Classical infectious bursal disease virus
CAM	Chorioallantoic membrane
DNA	Deoxyribonucleic acid
dpi	Day post inoculation
EID	Embryo infective dose
ELISA	Enzyme-linked immunosorbent assay
GD	Gumboro disease
HE	Hematoxylin and Eosin
HVR	Hypervariable region
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFN	Interferon
nVarIBDV	Novel variant infectious bursal disease virus
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
Rpm	Revolutions per minute
varIBDV	Variant infectious bursal disease virus

VP	Viral protein
vvIBDV	Very virulent infectious bursal disease virus
SW	Spleen weight
SW/BW	Spleen-to-body-weight ratio

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir.

PATOGENISITI VIRUS PENYAKIT BURSAL BERJANGKIT VARIAN NOVEL DALAM AYAM PEDAGING KOMERSIAL

Oleh

Chong Hao Han

2023

Penyelia: Profesor Dato' Dr Mohd Hair bin Bejo

Penyelia bersama: Dr Mazlina bin Mazlan

Penyakit bursal berjangkit (IBD) telah menyebabkan cabaran besar kepada industri ayam, menyebabkan kerugian ekonomi yang besar disebabkan oleh kesan kematian yang tinggi dan imunosupresif. Kemunculan varian baharu, varian novel virus penyakit bursal berjangkit (nVarIBDV), telah memburukkan keadaan, terutamanya kerana vaksin IBD yang sedia ada gagal memberikan kekebalan yang mencukupi terhadap strain ini. Kajian ini bertujuan untuk menyelidiki patogenisiti nVarIBDV dalam ayam pedaging komersial. Enam puluh anak ayam pedaging komersial

berumur tiga minggu dibahagikan kepada dua kumpulan: kumpulan nVarIBDV (28/60) dan kumpulan kawalan (32/60). Pada hari 0 selepas inokulasi (dpi), kumpulan nVarIBDV menerima inokulasi 1.0 mL yang mengandungi $10^{6.75}$ EID_{50}/0.1 mL nVarIBDV, diberikan melalui titisan mata (0.1 mL) dan secara oral (0.9 mL), manakala kumpulan kawalan kekal tidak dijangkiti. Sampel diambil dari kedua-dua kumpulan pada 0, 1, 3, 5, 7, 10, 14, dan 21 dpi, dengan hanya kumpulan kawalan yang diambil sampel pada hari ke-0 dpi. Tanda-tanda klinikal, berat badan, berat bursa, dan limpa diukur. Titer antibodi diperoleh melalui ELISA, dan analisis RT-qPCR dijalankan ke atas sampel dari bursa Fabricius, limpa, tonsil cekal, timus, dan sumsum tulang. Kedua-dua kumpulan tidak menunjukkan simptom klinikal tetapi terdapat impak negatif pada berat badan ayam yang diinokulasikan dengan nVarIBDV. Semasa nekropsi, kumpulan nVarIBDV menunjukkan atrofi bursa dan berat bursa yang berkurang, disokong oleh nilai BBIX di bawah 0.7 dari 3 hingga 21 dpi. Bursa Fabricius dalam kumpulan kawalan kelihatan normal, manakala kumpulan nVarIBDV menunjukkan lesi bursa sederhana hingga teruk, dengan skor yang signifikan ($p<0.05$) lebih tinggi daripada kumpulan kawalan. Analisis histopatologi bursa dalam kumpulan nVarIBDV mendedahkan degenerasi dan nekrosis folikel limfoid, infiltrasi inflammantori sel ke dalam ruang interstisial, epitelium yang tebal dan vakuol, dan pembentukan kista folikular. Tambahan pula, splenomegali diperhatikan pada 3 hingga 5 dpi pemaparan kepada jangkitan nVarIBDV. Titer antibodi IBD meningkat bermula dari 5 dpi, mencapai puncak yang sangat tinggi pada 10 dpi dan kekal tinggi sehingga 21 dpi berbeza dengan titer yang secara konsisten sangat rendah dalam kumpulan kawalan. Dalam analisis RT-qPCR, bursa ayam yang dijangkiti nVarIBDV menunjukkan beban virus tertinggi di antara organ yang dikaji, menunjukkan bursa sebagai organ sasaran utama bagi nVarIBDV. Kesimpulannya, nVarIBDV adalah patogenic dan menyebabkan penyakit subklinikal tanpa kematian dalam ayam

pedaging komersial dengan atrofi bursa yang teruk, splenomegali, respons antibodi yang tinggi dan peningkatan beban virus dalam organ.

Kata kunci: Penyakit bursal berjangkit, nVarIBDV, patogenisiti, ayam pedaging, atrofi bursa Fabricius, IBD antibodi, beban virus dalam organ

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 - Final Year Project.

PATHOGENICITY OF NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN COMMERCIAL BROILER CHICKENS

By

CHONG HAO HAN

2023

Supervisor: Professor Dato' Dr Mohd Hair bin Bejo

Co-supervisor: Dr Mazlina binti Mazlan

Infectious bursal disease (IBD) has presented significant challenges to the poultry industry, resulting in substantial economic losses due to high mortality and its immunosuppressive effects. The emergence of a new variant, novel variant infectious bursal disease virus (nVarIBDV), has exacerbated the situation, especially since existing IBD vaccines fail to provide adequate immunity against this strain. This study aimed to explore the pathogenicity of nVarIBDV in commercial broiler chickens. Sixty, three-week-old commercial broiler chicks were divided into two groups: the nVarIBDV group (28/60) and the control group (32/60). On 0 days post-inoculation (dpi), the

nVarIBDV group received a 1.0 mL inoculation containing $10^{6.75}$ EID_{50} of nVarIBDV, administered via eye drops (0.1 mL) and orally (0.9 mL), while the control group remained uninoculated. Samples were taken from both groups at 0, 1, 3, 5, 7, 10, 14, and 21 dpi, with only the control group sampled on 0 dpi. Throughout the study, clinical signs, body weight, bursa, and spleen weights were measured. IBD antibody titer was analysed through ELISA, and RT-qPCR analyses were conducted on samples from the bursa, spleen, caecal tonsil, thymus, and bone marrow. Neither group displayed clinical symptoms, but there was negative impact on body weight of chicken inoculated with nVarIBDV. Upon necropsy, the nVarIBDV group exhibited bursal atrophy and decreased bursal weight, supported by BBIX values below 0.7 from 3 to 21 dpi. The bursa of Fabricius (BF) in the control group appeared normal, while the nVarIBDV group displayed moderate to severe bursal lesions, with scores significantly ($p<0.05$) higher than the control group. The bursa histopathological analysis in nVarIBDV group revealed degeneration of lymphoid follicle, infiltration of inflammatory cells into interstitial space, thickened and vacuolated epithelium, and follicular cyst formation. Additionally, splenomegaly was observed from 3 to 5 dpi in response to nVarIBDV infection. The IBD antibody titer in nVarIBDV group increased starting from 5 dpi, reaching very high peak on 10 dpi and remained high until 21 dpi, in contrast to the consistently very low titer in the control group. In RT-qPCR analysis, the bursa of nVarIBDV-inoculated chickens exhibited the highest viral loads among the studied organs, highlighting the bursa as the primary target organ for nVarIBDV. In conclusion, the nVarIBDV is pathogenic and cause subclinical disease without mortality in commercial broiler chickens, body weight dropping, severe bursal atrophy, splenomegaly, high antibody response and increased viral loads in the organs.

Keywords: Infectious bursal disease, nVarIBDV, pathogenicity, broiler, atrophy of bursa of Fabricius, IBD antibody, viral load in the organs

1.0 INTRODUCTION

1.1 Background

Infectious bursal disease (IBD), also known as Gumboro disease, acquired its name from its initial identification in Gumboro, Delaware, back in 1957 (Cosgrove, 1962). This viral disease is caused by the IBD virus (IBDV) and primarily affects chickens within the age range of three to six weeks, leading to severe clinical symptoms. However, younger chickens 1 to 14-day-old are less susceptible due to the protection provided by maternal antibodies, while chickens older than 6 weeks tend to develop fewer clinical signs but exhibit high levels of antibody production (Mahgoub, 2012). IBDV has a high affinity for infecting the lymphoid tissue in the bursa of Fabricius (BF) (Mwenda et al., 2018). Consequently, IBD is characterized by bursal lesions resulting in atrophy, leading to immunosuppression in affected chickens (Orakpoghenor et al., 2020). The immunosuppression caused by IBD becomes a major concern as it makes chickens more vulnerable to other diseases, increasing their susceptibility to opportunistic pathogens. Additionally, other lymphoid organs, such as the spleen, thymus, and caecal tonsils, can also be affected by the virus. This widespread impact on the immune system poses significant health challenges for afflicted poultry, potentially leading to serious economic implications for the poultry industry.

IBD is caused by a highly contagious, non-enveloped, icosahedral, double-stranded RNA virus belonging to the Avibirnavirus genus within the Birnaviridae family (Chen et al., 2022). IBDV is composed of two segments of double-stranded RNA, referred to as segment A and segment B, both packaged within a single virus particle approximately 70 nm in diameter (Coulibaly et al., 2005). The IBDV genome encodes five viral proteins, including VP1 (90 kDa), VP2 (54 kDa), VP3 (28 kDa),

VP4 (25 kDa), and VP5 (21 kDa). Within segment A, there are two open reading frames (ORFs), with the larger ORF encoding a precursor polyprotein of 110 kDa (VP2-VP4-VP3). This precursor polyprotein undergoes co-translational processing and is cleaved into VP2, VP3, and VP4 by the auto-catalytic activity of VP4, which is a viral protease (Petit et al., 2000). Of all the components in the icosahedral capsid, VP2 plays a crucial role in determining cell tropism, antibody neutralization, virulence, and the pathogenic phenotype of virulent IBDV strains (Brandt et al., 2001). The smaller ORFs immediately preceding and partially overlapping the 110 kDa polyprotein gene encode viral protease 5 (VP5), which is not essential for virus replication. Segment B of the IBDV genome encodes viral polymerase 1 (VP1), which is important for viral replication and genetic evolution.

IBDV is classified into two main serotypes, with serotype I being pathogenic to chickens, while serotype II is isolated from turkeys and is nonpathogenic to chickens. IBDV serotype 1 primarily targets B lymphocytes in the BF, affecting the production of antibodies and leading to immunosuppression in the affected chickens. Therefore, infected birds become more susceptible to secondary diseases and vaccination failure (Fan et al., 2019). Serotype 1 can be further divided into three subtypes: classical IBDV (caIBDV), variant IBDV (varIBDV), and very virulent IBDV (vvIBDV) (Van et al., 2004). The caIBDV infection was first occurred in Gumboro in 1957 (Cosgrove, 1962). In 1985, the varIBDV strain was identified in Delaware, USA, and revealed that able to bypass the protection provided by classic strain vaccine (Chettle et al., 1989). Subsequently, vvIBDV emerged in Belgium, Europe, in 1989 and rapidly spread across Europe, Asia, Africa and South America (Di et al., 1999).

In 2017, nVarIBDV was identified and emerged in Eastern Asia, particularly in China (Zhang et al., 2022). Amino acid residue difference were detected in the HVR of the nVarIBDV (designated as LY21/2) viral protein 2 (VP2) compared to other IBDV strains, suspected to be a unique mutation (Huang et al., 2023). Additionally, it has been observed that LY21/2, as the major parent strain, was involved in a recombination event with another variant IBDV strain (16D69). This recombination event has contributed to significant changes in antigenic variation and viral pathogenicity, further highlighting the potential implications of genetic exchanges (Huang et al., 2023). In recent year, cases also reported in South Korea (Thai et al., 2021) and Japan (Myint et al., 2021). In Malaysia, the first case of nVarIBDV was reported in 2019 (Aliyu et al., 2022). It has been reported that nVarIBDV capable of inducing subclinical signs (Zhang et al., 2022) in chickens with body weight dropping (Fan et al., 2019). Studies have shown that nVarIBDV caused severe atrophy of BF, resulting in severe immunosuppression without causing mortality (Fan et al., 2020).

1.2 Justification

1. To determine the pathogenicity of nVarIBDV in commercial broiler chickens.
2. To develop new prevention and control strategies against nVarIBDV infections.
3. To prevent economic losses caused by nVarIBDV.

1.3 Hypothesis

H_0: There are no clinical signs, gross and histological lesions in commercial broiler chickens inoculated with nVarIBDV.

H_a: There are clinical signs, gross and histological lesions in commercial broiler chickens inoculated with nVarIBDV.

H_0: There is no increase of immune response in commercial broiler chickens inoculated with nVarIBDV.

H_a: There is increase of immune response in commercial broiler chickens inoculated with nVarIBDV.

H_0: There is no nVarIBDV in the bursa of Fabricius and other organs of commercial broiler chickens inoculated with nVarIBDV.

H_a: There is nVarIBDV in the bursa of Fabricius and other organs of commercial broiler chickens inoculated with nVarIBDV.

1.4 Objectives

1. To determine the clinical signs, gross and histological lesions in commercial broiler chickens inoculated with nVarIBDV.
2. To determine the immune response of commercial broiler chickens inoculated with nVarIBDV.
3. To determine presence of the nVarIBDV in the bursa of Fabricius and other organs of chickens inoculated with nVarIBDV.

2.0 LITERATURE REVIEW

2.1 Background

In 2017, a novel variant IBDV (nVarIBDV), with Genotype A2dB1, emerged in Eastern Asia, particularly in China. This novel isolate was termed the antigenic variant of IBDV (varIBDV) (Statts, 2020). According to Jackwood et al. (2018), varIBDVs were identified as Genogroup 2, and they classified them into three sub-lineages with an American origin. Based on the results of HVR VP2 phylogenetic analysis and key amino acid comparisons among seven IBDV genogroups, researchers found that two Korean varIBDV strains (19D51 and 19D69) and certain novel varIBDV strains isolated in China were also classified under Genogroup 2. This discovery led to the establishment of a new sub-lineage, G2d, in the phylogenetic tree topology, thus defining the novel variant group. The novel variant strain consists of several amino acid substitutions that significantly influenced its antigenicity, pathogenicity, and interaction with the host immune response as compared to other G2 sub-lineages (Thai et al., 2021).

This nVarIBDV able to induce subclinical symptoms in chickens, posing a significant threat to the poultry industry (Zhang et al., 2022). In recent years, nVarIBDV has also been reported in other countries such as South Korea (Thai et al., 2021), Japan (Myint et al., 2021), and Malaysia (Aliyu et al., 2021). The nVarIBDV exhibits high morbidity and causes serious regression of the BF. Despite vaccination with three types of IBD vaccines against vvIBDV (attenuated live vaccine, subunit vaccine and combined vaccine), nVarIBDV has demonstrated remarkable resilience, managing to severely damage the BF and other critical immune organs in affected chickens (Fan et al., 2020).

According to studies, nVarIBDV causes subclinical infection with evident body weight dropping. The bursa exhibits signs of atrophy, hemorrhages, and a yellowish appearance due to inflammatory exudation. Furthermore, splenomegaly is initially observed, but at later stages, spleen atrophy occurs around 25 days post-inoculation (dpi) (Fan et al., 2019). Histologically, the BF undergoes significant changes due to nVarIBDV infection. These changes include the destruction of lymphocytes, minimal to no inflammatory response, severe follicular atrophy, infolding epithelium into damaged follicles, and proliferation of fibrous tissues. Moreover, the decrease in lymphocyte numbers resulting from their destruction leads to a reduction in the production of cytokines.

2.2 Pathogenesis

Under natural conditions, chickens can acquire IBDV through various routes, including indirect exposure via contaminated water and feed, as well as direct exposure through conjunctival membranes (ocular route) to contaminated droplets or dust (Gilchrist, 2005). Once the virus enters the chicken's body, it primarily infects and replicates within gut-associated macrophages. Infected macrophages then enter the bloodstream, leading to primary viremia and carrying the virus to the BF. Within the BF, intracytoplasmic replication of the virus occurs specifically in IgM+ B lymphocytes (Hiraga et al., 1994). In addition, the infection triggers an immune response, resulting in the production of interferon-8 (IFN-8) and the release of inflammatory cytokines. These cytokines exacerbate the condition of the bursa, further impacting its function (Kim et al., 1998). Moreover, IFN induces apoptosis in infected and surrounding healthy B-cells (Lam, 1997). As the infection progresses, the virus enters the bloodstream again, leading to secondary viremia. This allows the virus to spread and cause damage to other tissues and organs, such as the

spleen, caecal tonsils, and thymus of the chickens (Mahgoub, 2012). During the early stages of infection (2 dpi), there is inflammation of the BF. By 3 to 4 dpi, cytolytic changes are observed in all infected bursal IgM+ B cells (Cheville, 1967). Subsequently, between 7 to 21 dpi, there is a significant reduction in the population of IgM+ B cells in the bursa, leading to severe immunosuppression and making the chickens more susceptible to secondary infections (Petkov et al., 2009).

2.3 Pathogenicity of novel variant IBDV isolate

According to studies, chickens inoculated with nVarIBDV did not show any clinical symptoms or mortality. However, their body weight was negatively affected, indicating an impact on their overall health and growth (Huang et al., 2023). During necropsy, it was observed that the SHG19 strain of nVarIBDV caused severe damage to the BF at 5 dpi and 10 dpi. The affected bursa exhibited signs of atrophy, inflammatory yellowish exudate staining, and a hard texture (Huang et al., 2023). On the other hand, no obvious gross lesions were detected in the bursa of chicken carcasses infected with other strains of IBDV (Thai et al., 2021). Huang et al. (2023) reported that the SHG19 strain caused lymphocyte reduction, macrophage infiltration, connective tissue hyperplasia, atrophy, and destruction of follicles in the BF, even in vaccinated chickens. As early as 1 dpi, a marked decrease in lymphocyte numbers and infiltration of macrophages in the follicles were observed (Fan et al., 2019). This was followed by the appearance of vacuoles and cystic cavities within the lymphoid follicles (Thai et al., 2021). From 3 dpi, the proliferation of fibrous tissue was observed around the follicle. By 5 dpi, the follicle was severely atrophied (Fan et al., 2019).

3.0 MATERIALS AND METHODS

3.1 Experimental Design

A total of 60 commercial broiler chickens, aged 3 weeks, were randomly assigned to two groups: nVarIBDV (28/60) and control (32/60). On 0 dpi, the chickens in the nVarIBDV group were inoculated with 1.0 mL of nVarIBDV, containing $10^{6.75}$ EID_{50} / 1.0 mL, administered via 0.1 mL eye drops and 0.9 mL orally. The control group did not receive any inoculation. Feed and water were provided *ad libitum*, and clinical signs were monitored at least twice daily. Any deaths or severely sick chickens were sacrificed for sampling throughout the trial. Samples were taken from both groups at 0, 1, 3, 5, 7, 10, 14, and 21 dpi, with only the control group sampled on 0 dpi. Four chickens from each group were sacrificed for sampling at each time point. The body weight of selected chickens was measured, and serum samples were collected for IBD antibody detection using the Enzyme-linked immunosorbent assay (ELISA) technique. During necropsy, gross lesions were recorded, and the weights of the bursa and spleen were measured. The bursa-to-body-weight ratio was calculated using the formula [Bursa-to-body-weight ratio (B/BW) = (bursa weight / body weight) x 1000]. The spleen-to-body-weight ratio was calculated using the formula [Spleen-to-body-weight ratio (SW/BW) = (spleen weight / body weight) x 1000]. To assess the atrophy of the BF, the BF:body weight index (BBIX) was computed using the formula [BBIX = (BF:body weight ratio)/(BF:body weight ratio in the negative group)]. A BBIX value below 0.70 indicated atrophy of the bursa (Lucio and Hitchner, 1979). Samples of the BF were collected and fixed in 10% neutral-buffered formalin for histopathological examination. Samples of the BF, spleen, caecal tonsil, thymus, and bone marrow were collected for virus detection using the polymerase chain reaction (PCR) technique.

3.2 nVarIBDV titration

The virus titration method was used to determine the concentration of nVarIBDV in a suspension. This process involved a series of ten-fold dilutions conducted on the suspension to accurately measure the viral content. Initially, ten dilution tubes were prepared and labeled sequentially from 10^{-1} to 10^{-10}. Each tube was filled with 900 µl of Phosphate Buffered Saline (PBS). Subsequently, 100 µl of the nVarIBDV suspension was added to the first tube labeled 10^{-1} and thoroughly mixed, resulting in a one-tenth concentration of the original suspension. Then, 100 µl of the 10^{-1} dilution was further mixed with 900 µl of PBS in the second tube (10^{-2} dilution), and this process was repeated until a 10^{-10} dilution was achieved. These ten-fold dilutions allowed for a progressive decrease in virus concentration. To evaluate the infectivity of each dilution, five embryonated eggs were prepared for each dilution, totaling 50 eggs overall. Using separate needles and syringes for each dilution, the inoculum was administered into the eggs, which were then incubated at 37°C for 24 hours. The eggs were subsequently monitored for mortality rates over the next seven days. Embryonated eggs that died within 24 hours were not harvested as inoculum. The Reed and Muench mathematical technique was used to establish the dilution by calculating the index using the formula (Reed & Muench, 1938): $Index = \frac{(\%\ infected\ at\ dilution\ immediately\ above\ 50\%) - 50\%}{(\%\ infected\ at\ dilution\ immediately\ above\ 50\%) - (\%\ infected\ at\ dilution\ immediately\ below\ 50\%)}$.

This index was then applied to the dilution that resulted in the percentage of infection immediately above 50 percent. The reciprocal of this dilution provides the number of Egg Infective Dose 50 (EID_{50}) in the 0.1 mL volume of inoculum administered into each egg. By multiplying this number by ten, the EID_{50} in 1 mL of the original inoculum can be determined.

3.3 nVarIBDV inoculum preparation

The chorioallantoic membrane (CAM) was harvested, pooled, and weighed from the infected embryonated eggs at each dilution. Subsequently, the CAM pools were adequately diluted with phosphate-buffered saline (PBS) at a ratio of 1 part CAM to 2 parts PBS. To homogenize the mixture, tissue grinders were used, ensuring a consistent and thorough blending of the CAM samples with the PBS. The homogenates were then subjected to centrifugation at 1500 revolutions per minute (rpm) for 5 minutes. This process led to the separation of the liquid portion, which was discarded. The virus-containing supernatant was carefully collected from the centrifuged samples and filtered through a syringe filter into a separate sterile tube. The filtered contents now contained the virus and were referred to as the inoculum. It was essential to ensure that the inoculum contained the correct amount of the virus before proceeding with further experiments, and as such, a virus titer check was performed to guarantee the accuracy and reliability of the inoculum.

3.4 IBD Antibody Titre (ELISA)

Serum samples from chickens at different time points on 0, 1, 3, 5, 7, 10, 14, and 21 dpi were collected for quantifying IBD antibody titer using a commercial BioChek Antibody enzyme-linked immunosorbent assay (ELISA) test kit. The serum samples were diluted at a ratio of 1:500 (v/v). Each well of the ELISA plate was filled with 100 µL of dilution buffer, followed by the addition of 100 µL of the appropriately diluted serum. Separate wells were allocated for positive and negative controls with the same volume. The ELISA plate was covered and incubated at room temperature for one hour to facilitate immune reactions. Dilution Plate 1, containing 96 wells, was prepared, and 5 µL of the sample was added to each well. Dilution Plate 2 was prepared with 297 µL of green diluent. Subsequently, 245 µL of diluent was added to

Dilution Plate 1. Then, 33 µL from Dilution Plate 1 was transferred to Dilution Plate 2. Afterward, 100 µL from Dilution Plate 2 was dispensed into the coating plate, which was incubated for 30 minutes. Following incubation, the wells were emptied and washed four times using approximately 300 µL of wash solution to remove any unbound substances. Subsequently, 100 µL of the conjugate was added to each well, and the plate was incubated at 37℃ for 30 minutes. The wells were emptied and washed four times with washing buffer to remove excess conjugate. Next, 100 µL of substrate was added to each well and incubated for 15 minutes in the dark. Finally, the reaction was stopped by adding 100 µL of stop solution to each well. A microtitre plate reader was used to measure the absorbance at 405 nm within 5 minutes after the addition of the stop solution, allowing for the quantification of antibody titers in the chicken serum samples.

3.5 Viral RNA extraction and RT-qPCR

Pooled samples, including bursa, spleen, thymus, caecal tonsil, and bone marrow, were collected in a sterile buffer and incubated for 24 hours for Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. Subsequently, the samples were thoroughly washed using pulse-vortexing, and the resulting supernatant containing genetic material was utilized. Each purification process used twenty milligrams of tissue. To initiate purification, 200 µl of lysis solution was introduced into a Kylt® Lysis tube, followed by the addition of 200 µl of the sample. Ten µL of liquid proteinase K was then introduced into the lysis tube. The mixture was pulse-vortexed for 20 seconds and centrifuged for 5 seconds. This mixture was subsequently incubated at room temperature for 5 minutes and then at 70°C for an additional 5 minutes, before being allowed to cool for 2 minutes. For the binding step, 200 µL of 96% ethanol was added to the mixture, which was pulse-vortexed and

centrifuged for 5 seconds. The resulting mixture was transferred to a Kylt® Binding column and centrifuged for 1 minute at 10,400 rpm. Following this step, the initial collection tube was replaced with a new one. An additional 500 µL of Wash Solution 1 was added to the Kylt® Binding column and centrifuged for 1 minute at 10,400 rpm, with subsequent replacement of the collection tube. This wash step was repeated using 500 µL of Wash Solution 2, followed by centrifugation at maximum speed (14,000 rpm) for 2 minutes. The collection tube was again discarded, and the column was placed on the elution tube. For elution, 100 µL of elution buffer was directly added to the membrane without any physical contact. The mixture was then incubated for 1 minute at 70°C and subsequently centrifuged for 1 minute at maximum speed. The Kylt® Binding column was then discarded, and the elution tube was sealed. The resulting eluate contained the purified RNA and DNA. Following nucleic acid extraction, an RT-qPCR assay was conducted utilizing specific primers to amplify the purified nucleic acid and quantify the viral DNA copies present in the samples.

3.6 Histopathology and Lesion Scoring

The BF samples were collected and fixed in 10% neutral buffered formalin. These tissue samples were then cross-sectionally trimmed to a thickness of 5mm and subjected to an automated processing machine (Leica ASP 300) for 24 hours. The processing procedure encompassed various stages, including dehydration, clearing and wax infiltration, embedding and microtomy, deparaffinization, staining with hematoxylin and eosin, and finally cover slipping. Following this comprehensive processing, the resultant slides were observed under a light microscope to identify any microscopic alterations. The assessment of lesions was performed using a grading scale ranging from 0 (normal), 1 (mild), 2 (mild to moderate), 3 (moderate), 4 (moderate to severe), 5 (severe) (Hair-Bejo et al., 2000; Appendix 1).

3.8 Statistical analysis

Data collected were analyzed using SPSS version 28.0. With a confidence interval of 95%, statistical results were considered significant when $p < 0.05$.

4.0 Results

4.1 Clinical signs

There was no clinical manifestation observed over the entire 21-day trial period (Figure 1).

Figure 1: Normal clinical signs of chickens. (a) Control group, day 0 dpi, and nVarIBDV groups on: (b) 1dpi, (c) 3 dpi, (d) 5 dpi, (e) 7 dpi, (f) 10 dpi, (g) 14 dpi and (h) 21 dpi.

4.2 Body Weight

The body weights of both the control group and the nVarIBDV group showed overall increased from 0 dpi to 21 dpi. On 5 dpi, there was statistically significant difference ($p < 0.05$) between the two groups, with the nVarIBDV group displaying a significantly lower body weight compared to the control group. Conversely, on 1, 3, 7, 14, and 21 dpi., there were no significant differences ($p > 0.05$) in body weight between the two groups (Figure 2; Appendix 2).

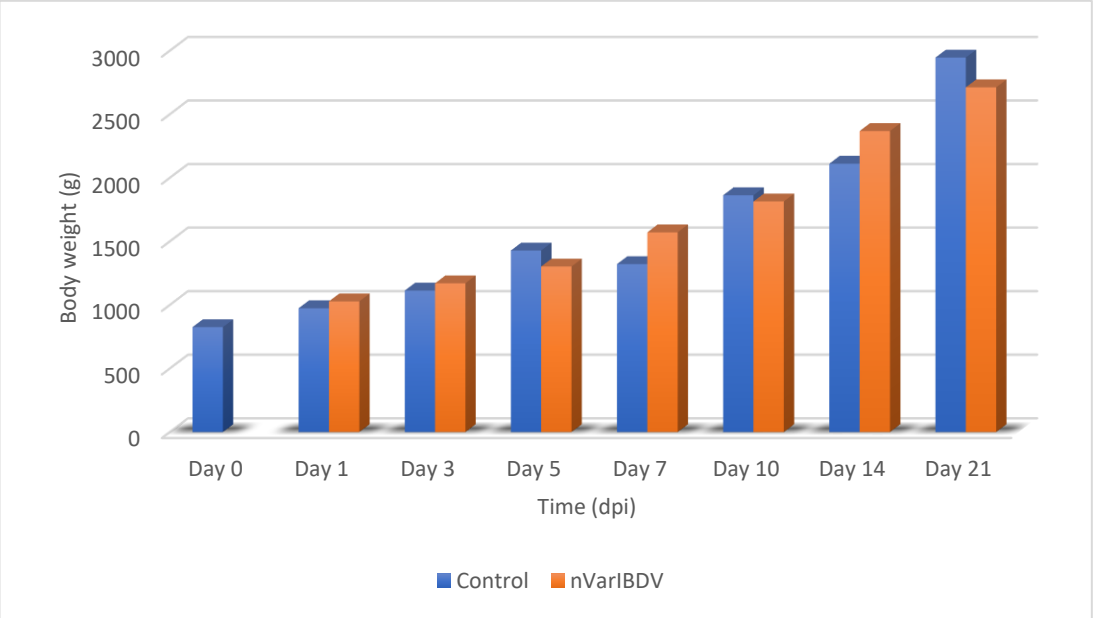

Figure 2: Body weight of chickens throughout the study.

4.3 Bursa Weight

At the intervals of 1, 3, and 14 dpi, there were no statistically significant differences ($p > 0.05$) in bursa weight between the control group and the nVarIBDV group. However, on 5, 7, 10, and 21 dpi, there were statistically significant differences in bursa weight between the nVarIBDV and control groups. Specifically, the bursa weight of the nVarIBDV group was significantly lower ($p < 0.05$) than that of the control group during these time points (Figure 3; Appendix 3).

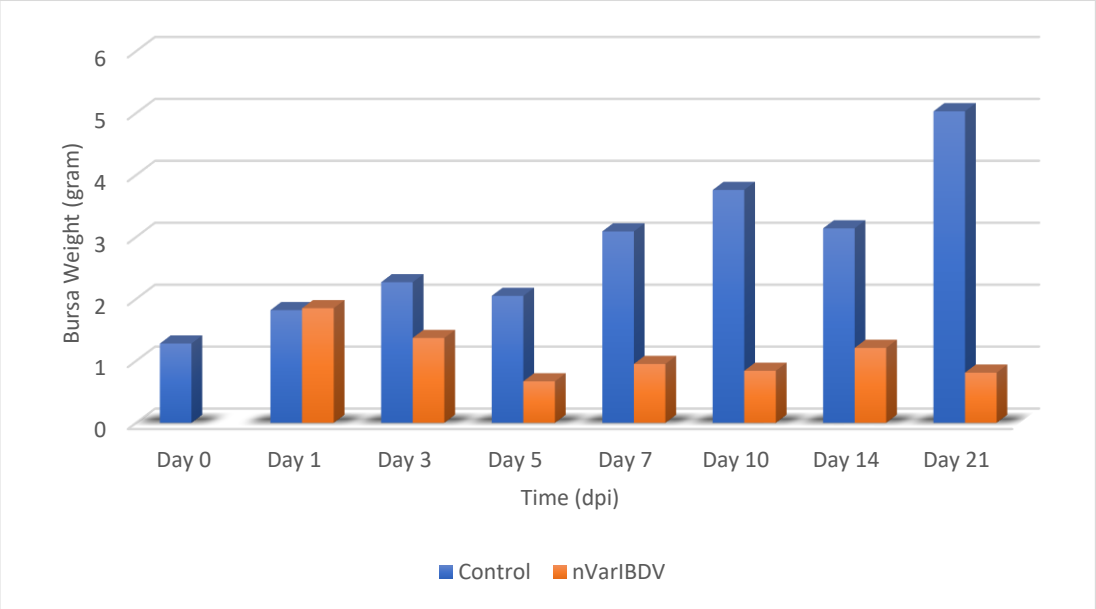

Figure 3: Bursa of Fabricius weight of chickens throughout the study.

4.4 Bursa-to-Body-Weight ratio

There were no significant differences ($p > 0.05$) observed in the bursa-to-body-weight ratio between the two groups on 1 and 3 dpi. However, on the subsequent sampling on 5, 7, 10, 14, and 21 dpi, the bursa-to-body-weight-ratio of chickens in the nVarIBDV group was significantly lower ($p < 0.05$) than that of the control group (Figure 4; Appendix 4).

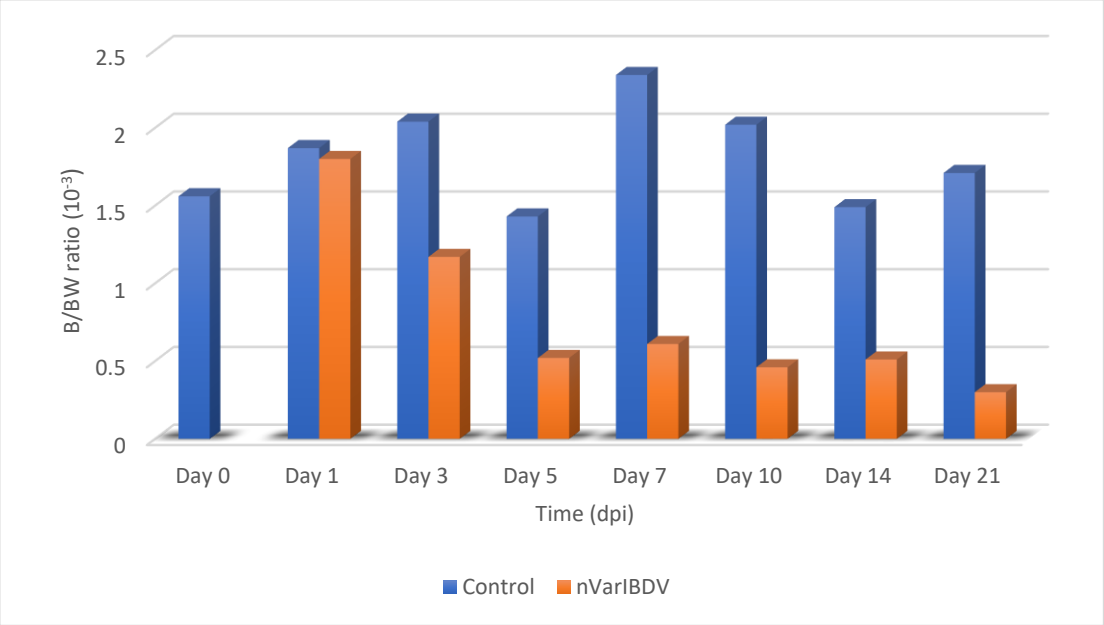

Figure 4: Bursa-to-body-weight ratio of chickens throughout the study.

4.5 BBIX Index

The BBIX index that below than 0.7 indicates bursal atrophy (Lucio and Hitchner, 1979). From 3 to 21 dpi, the BBIX were lower than 0.7, acknowledged that bursal atrophy was occurring (Figure 5).

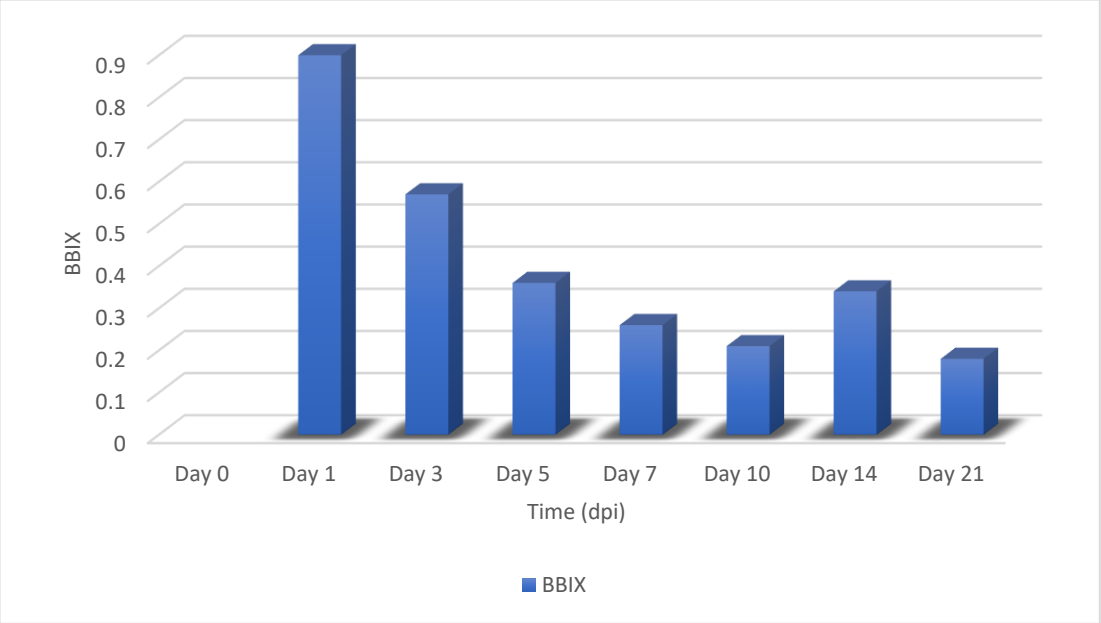

Figure 5: BBIX of chickens throughout the study.

4.6 Spleen Weight

The spleen weights of chickens in both the nVarIBDV and control groups showed no significant differences ($p > 0.05$) on 1, 7, 10, 14, and 21 dpi. However, on 3 and 5 dpi, there were significant differences in spleen weight between the control group and nVarIBDV groups. The spleen weight of chickens in the nVarIBDV group was significantly higher ($p < 0.05$) than that of the control group on 3 and 5 dpi (Figure 6; Appendix 5).

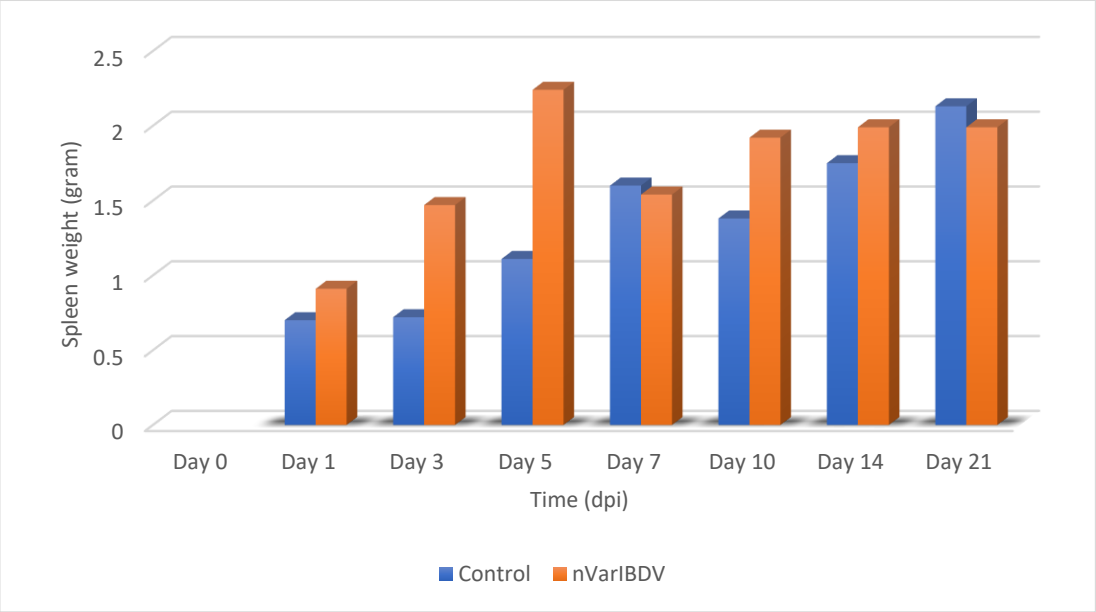

Figure 6: Spleen weight of chickens throughout the study.

4.7 Spleen-to-Body-Weight ratio

The spleen-to-body-weight ratio of chickens in the nVarIBDV group showed statistically significant increase ($p < 0.05$) compared to the control group on 3 and 5 dpi. There were no significant differences in the spleen-to-body-weight ratio between the control and nVarIBDV groups on 1, 7, 10, 14, and 21 dpi (Figure 7; Appendix 6).

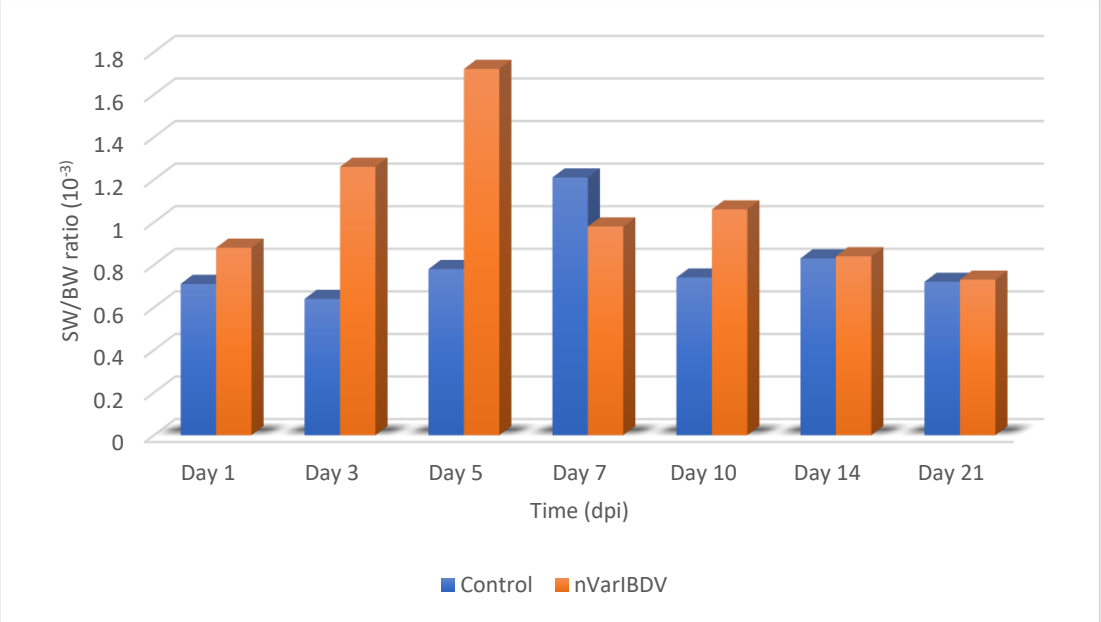

Figure 7: Spleen-to-body-weight ratio of chickens throughout the study.

4.8 Gross lesions

4.8.1 Bursa

The bursa of Fabricius of chickens in nVarIBDV group were atrophied gradually from 5 dpi until 21 dpi. (Figure 8 to 14).

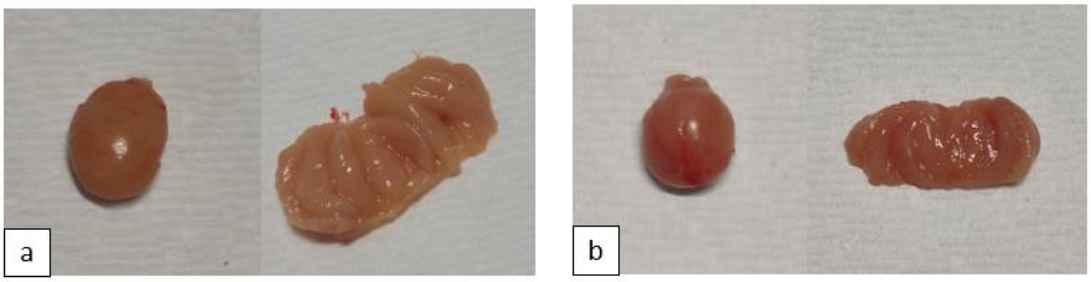

Figure 8: Normal bursa of Fabricius in chickens on 1 dpi. (a) Control group and (b) nVarIBDV group.

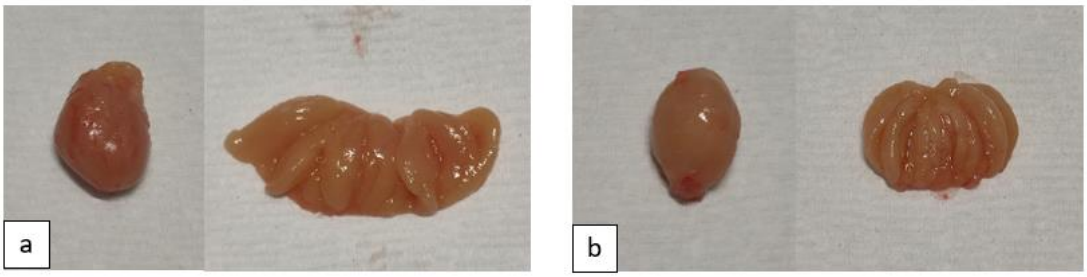

Figure 9: Normal bursa of Fabricius in chickens on 3 dpi. (a) Control group and (b) nVarIBDV group.

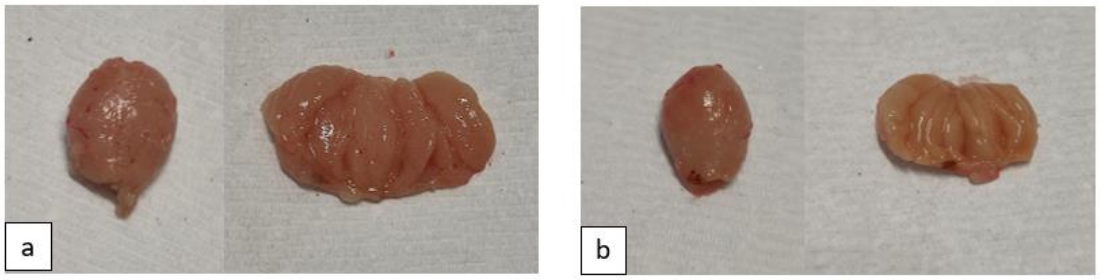

Figure 10: The bursa of Fabricius in chickens on 5 dpi. (a) Control group, normal and (b) nVarIBDV group, atrophy.

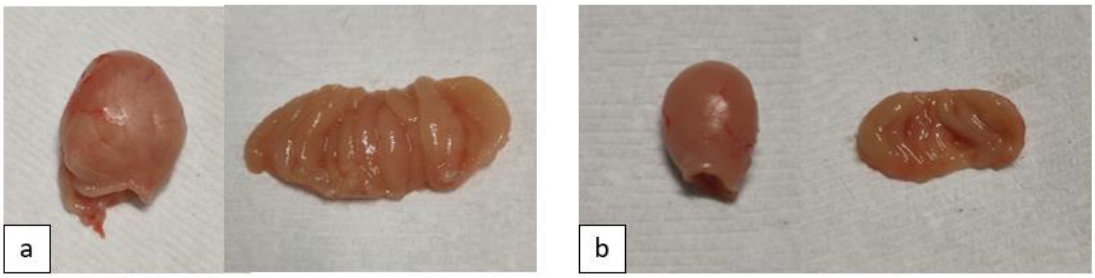

Figure 11: The bursa of Fabricius in chickens on 7 dpi. (a) Control group, normal and (b) nVarIBDV group, atrophy.

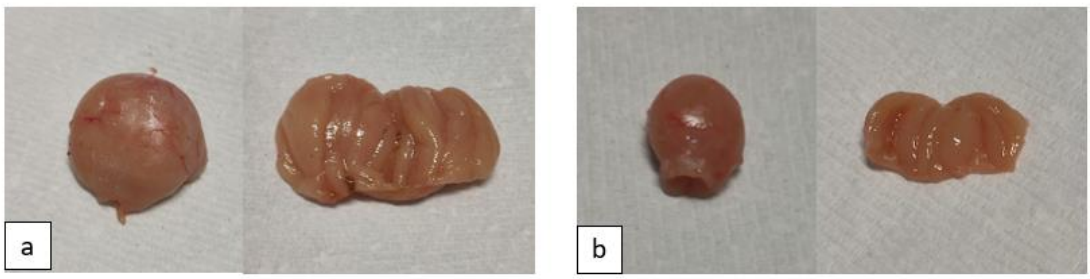

Figure 12: The bursa of Fabricius in chickens on 10 dpi. (a) Control group, normal and (b) nVarIBDV group, atrophy.

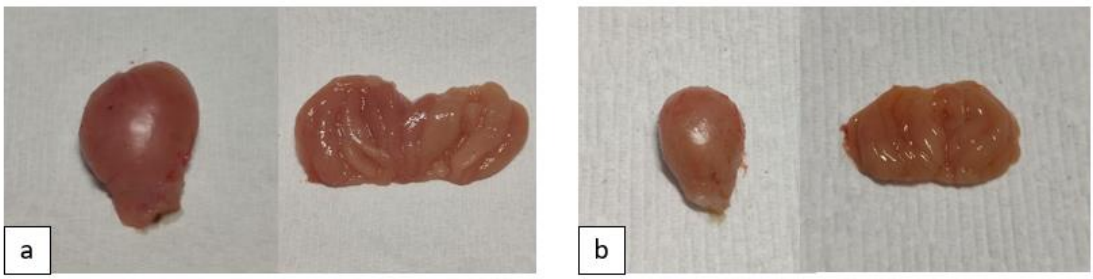

Figure 13: The bursa of Fabricius in chickens on 14 dpi. (a) Control group, normal and (b) nVarIBDV group, atrophy.

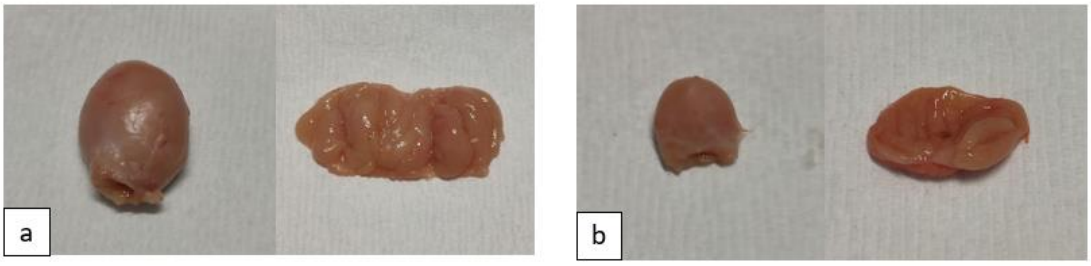

Figure 14*:* The bursa of Fabricius in chickens on 21 dpi. (a) Control group, normal and (b) nVarIBDV group, atrophy.

4.8.2 Spleen

The spleens of chickens from the control group were normal without gross lesions throughout the 21 days of the study. The spleen of chickens from the nVarIBDV group was enlarged on 3 and 5 dpi, returning to normal size from 7 dpi (Figure 15 to 21).

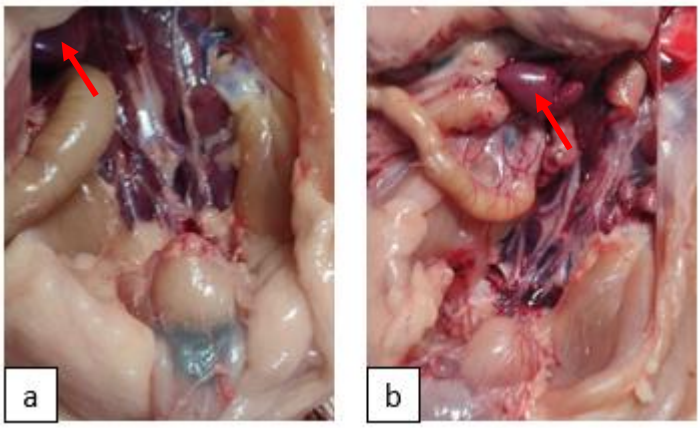

Figure 15: Normal spleen (red arrow) of the chickens on 1 dpi. (a) Control group and (b) nVarIBDV group.

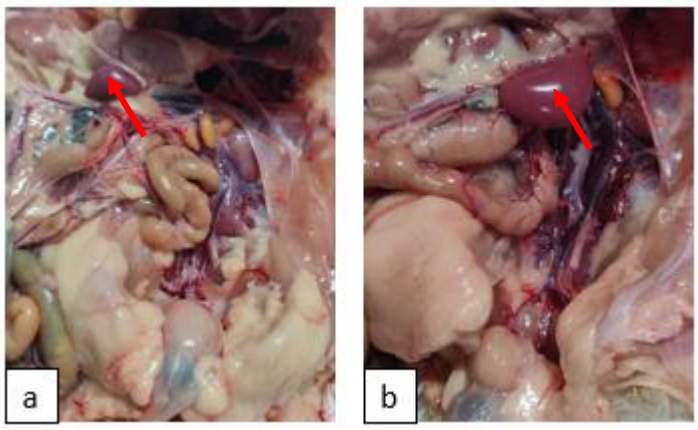

Figure 16: The spleen of the chickens (red arrow) on 3 dpi. (a) Control group and (b) nVarIBDV group, enlarged.

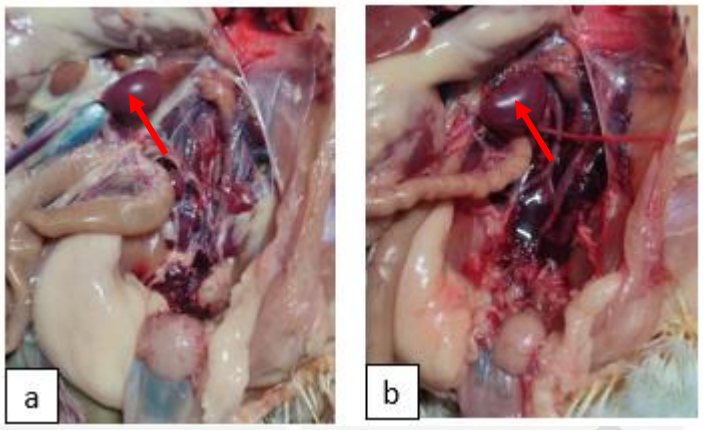

Figure 17: The spleen of the chickens (red arrow) on 5 dpi. (a) Control group and (b) nVarIBDV group, enlarged.

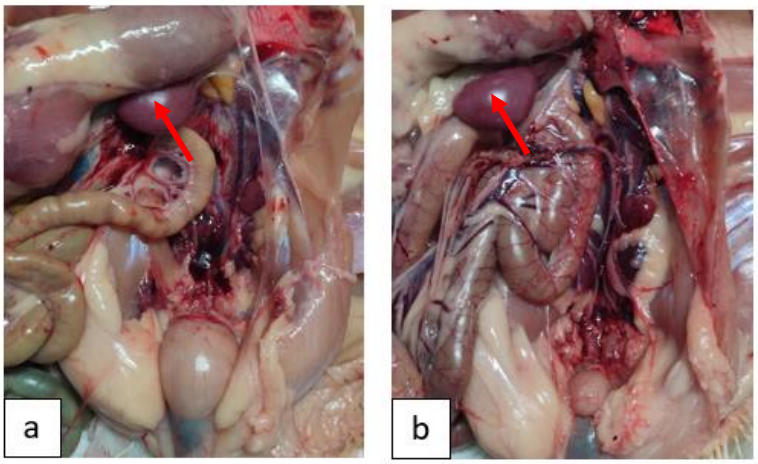

Figure 18: Normal spleen of the chickens (red arrow) on 7 dpi. (a) Control group and (b) nVarIBDV group.

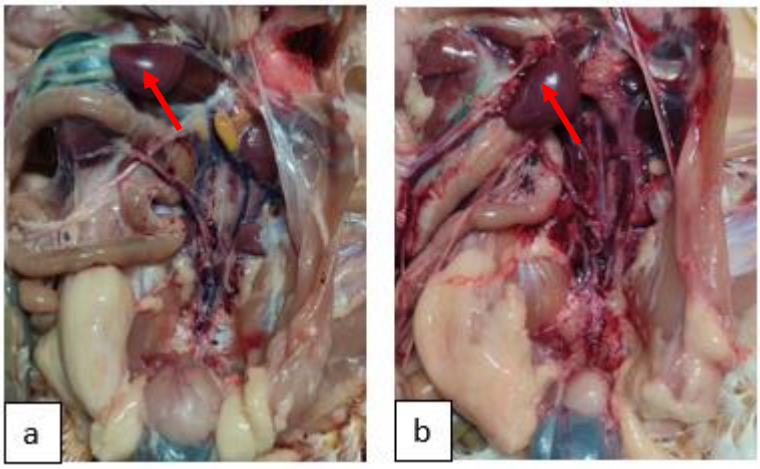

Figure 19: Normal spleen of the chickens (red arrow) on 10 dpi. (a) Control group and (b) nVarIBDV group.

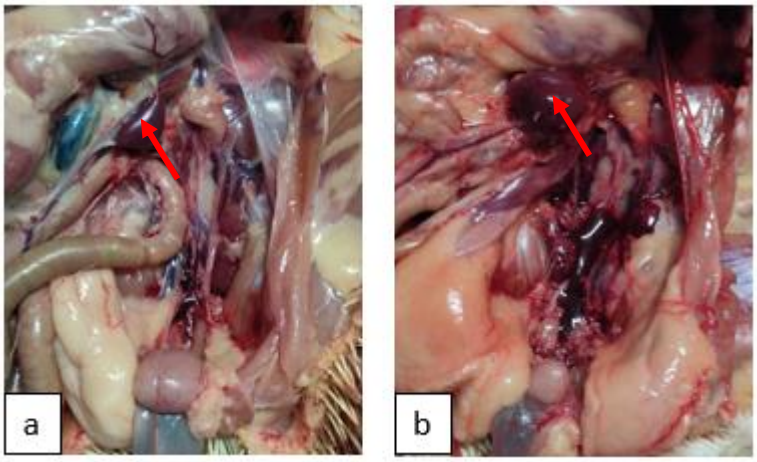

Figure 20: Normal spleen of the chickens (red arrow) on 14 dpi. (a) Control group and (b) nVarIBDV group.

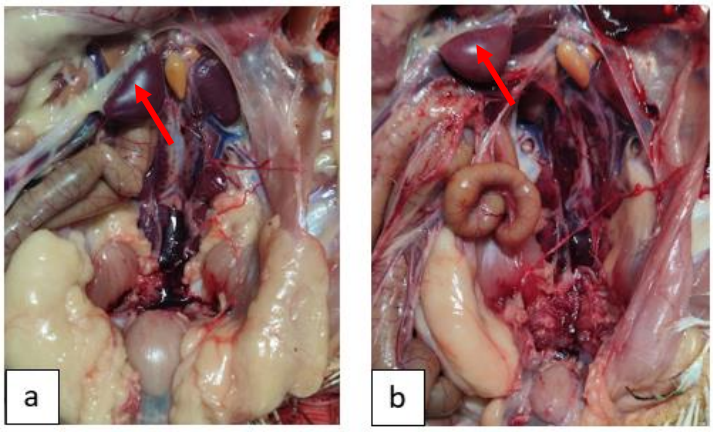

Figure 21: Normal spleen of the chickens (red arrow) on 21 dpi. (a) Control group and (b) nVarIBDV group.

4.8.3 Thymus

There was no gross lesion in the thymus of chickens from control and nVarIBDV group throughout the trial (Figure 22).

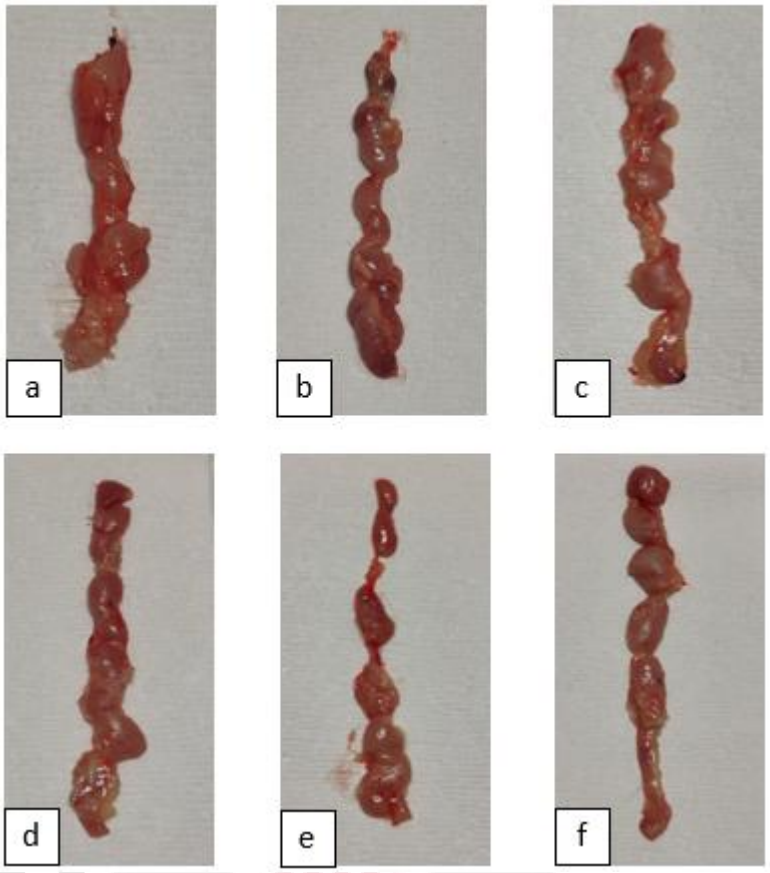

Figure 22: No abnormal gross lesion of thymus of the chickens. (a) Control group and nVarIBDV groups on: 1 dpi (b) 3 dpi (c) 7 dpi (d) 10 dpi (e) 14 dpi and (f) 21 dpi.

4.8.4 Muscle

There was no gross lesion in the muscle of chickens from control and nVarIBDV group throughout the trial (Figure 23).

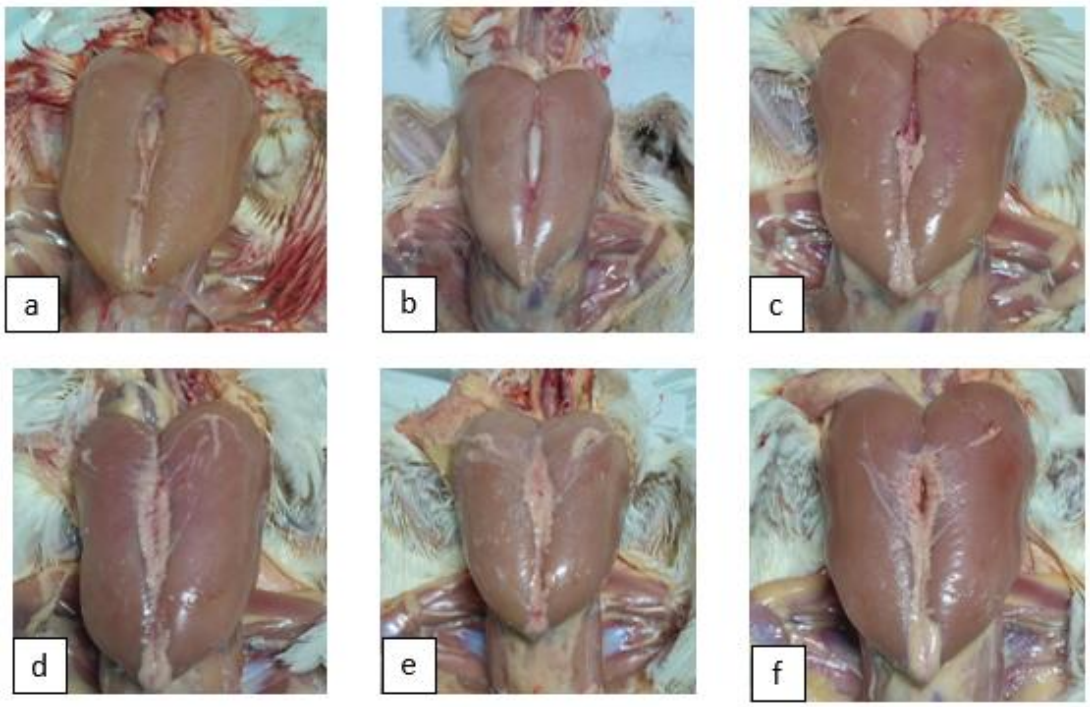

Figure 23: No abnormal gross lesion of muscle of the chickens. (a) Control group and nVarIBDV groups on: 1 dpi (b) 3 dpi (c) 7 dpi (d) 10 dpi (e) 14 dpi and (f) 21 dpi.

4.8.5 Liver

There was no gross lesion in the liver of chickens from control and nVarIBDV group throughout the trial (Figure 24).

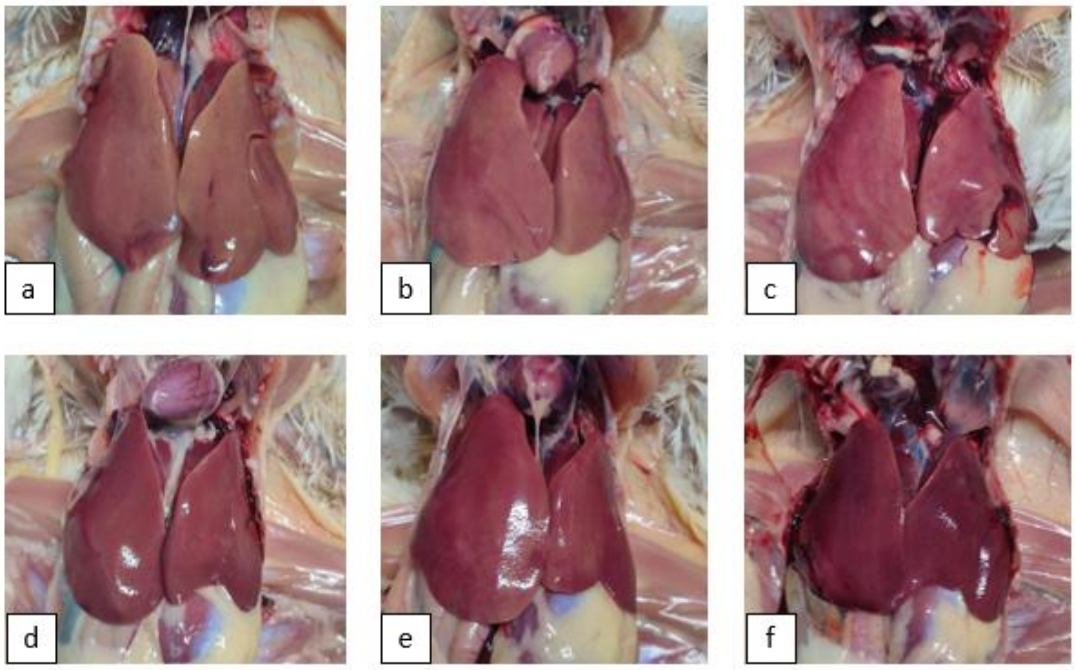

Figure 24: No abnormal gross lesion of liver of the chickens. (a) Control group and nVarIBDV groups on: 1 dpi (b) 3 dpi (c) 7 dpi (d) 10 dpi (e) 14 dpi and (f) 21 dpi.

4.9 Bursa lesion scoring (0-5)

The bursa lesion scoring in both the control and nVarIBDV groups did not show a significant difference on 1 dpi. However, there was significant difference ($p < 0.05$) in bursal lesion scoring between the control and nVarIBDV groups from 3 to 21 dpi. During this period, the bursal lesion scoring in the nVarIBDV group was significantly higher than that in the control group (Figure 25; Appendix 7).

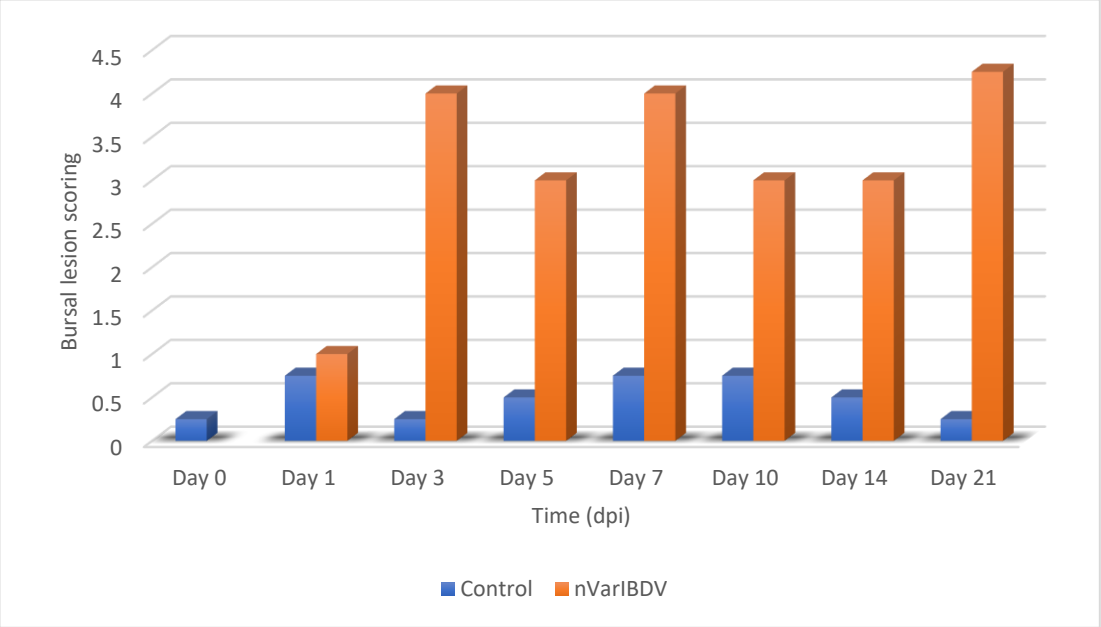

Figure 25: Bursal lesion scoring of the chickens throughout the study.

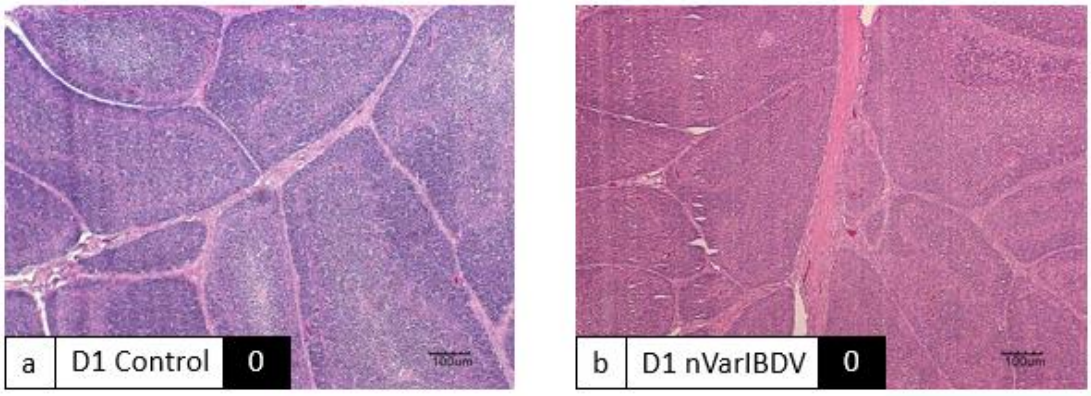

Figure 26: Histological bursal lesion scoring of the chickens on 1 dpi. (a) Control group. normal bursa, score 0, (b) nVarIBDV group, score 1. HE, Bar = 100µm.

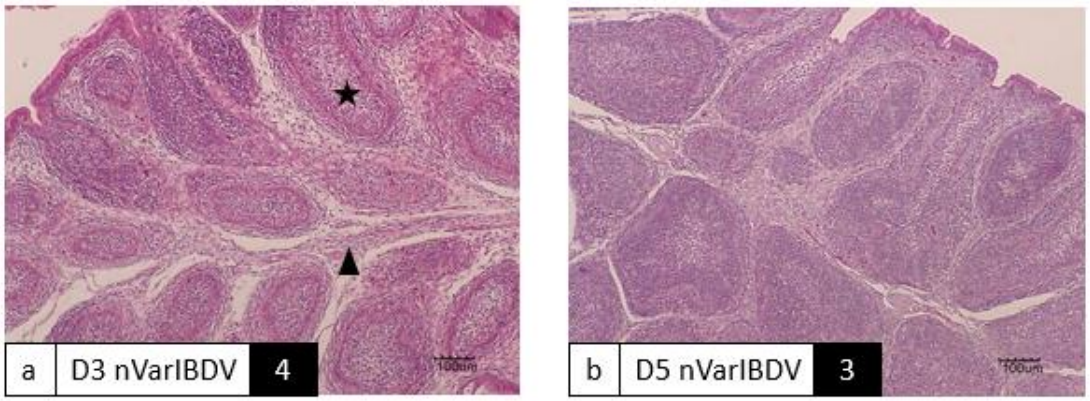

Figure 27: Histological bursal lesion scoring of the chickens from nVarIBDV groups on: (a) 3 dpi: score 4, moderate to severe lymphoid depletion (star), bursal follicular atrophy, thickened interstitial connective tissue with inflammatory cells infiltration (arrowhead) and (b) 5 dpi: score 3, moderate lymphoid depletion. HE, Bar = 100µm.

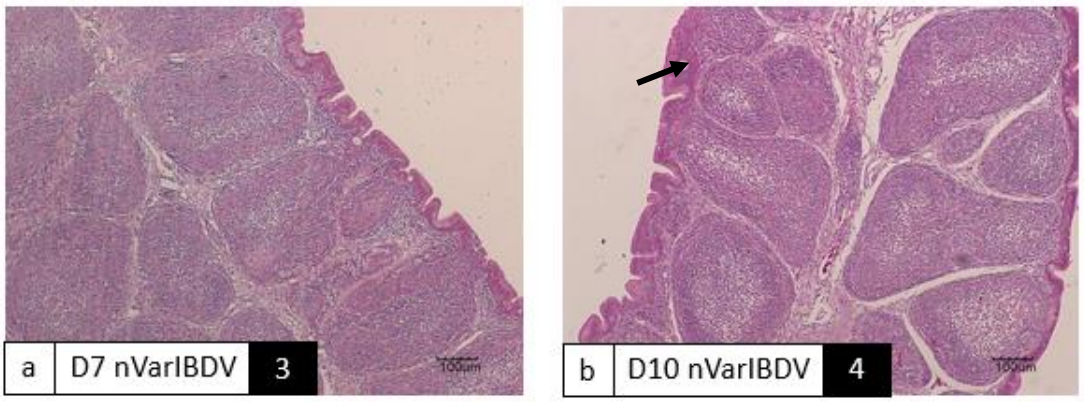

Figure 28: Histological bursal lesion scoring of the chickens from nVarIBDV groups on: (a) 7 dpi: score 3, moderate lymphoid depletion and (b) 10 dpi: score 3, moderate lymphoid cells depletion in follicles, thickened and vacuolated epithelial lining (arrow). HE, Bar = 100µm.

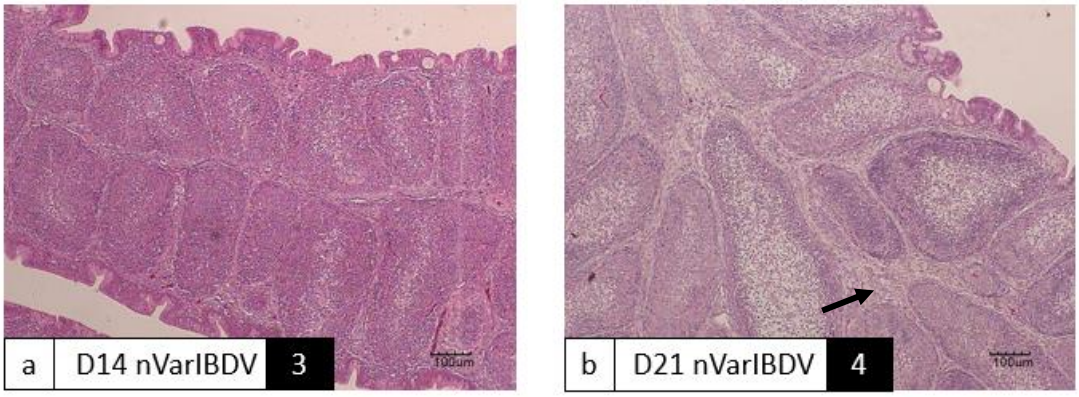

Figure 29: Histological bursal lesion scoring of the chickens from nVarIBDV groups on: (a) 14 dpi: score 3, moderate lymphoid depletion and (b) 21 dpi: score 4, moderate to severe lymphoid depletion, bursal follicular atrophy, proliferation of fibrous tissue (arrow). HE, Bar = 100µm.

4.10 IBD antibody titre

The IBD antibody titers in the nVarIBDV group showed a consistent upward trend over the course of 21 days. On 1 and 3 dpi, there was no statistically significant difference ($p > 0.05$) in IBD antibody titer between the control group and the nVarIBDV group. The IBD antibody titer of nVarIBDV was significantly higher ($p < 0.05$) than the control group starting from 5 to 21 dpi (Figure 28; Appendix 8).

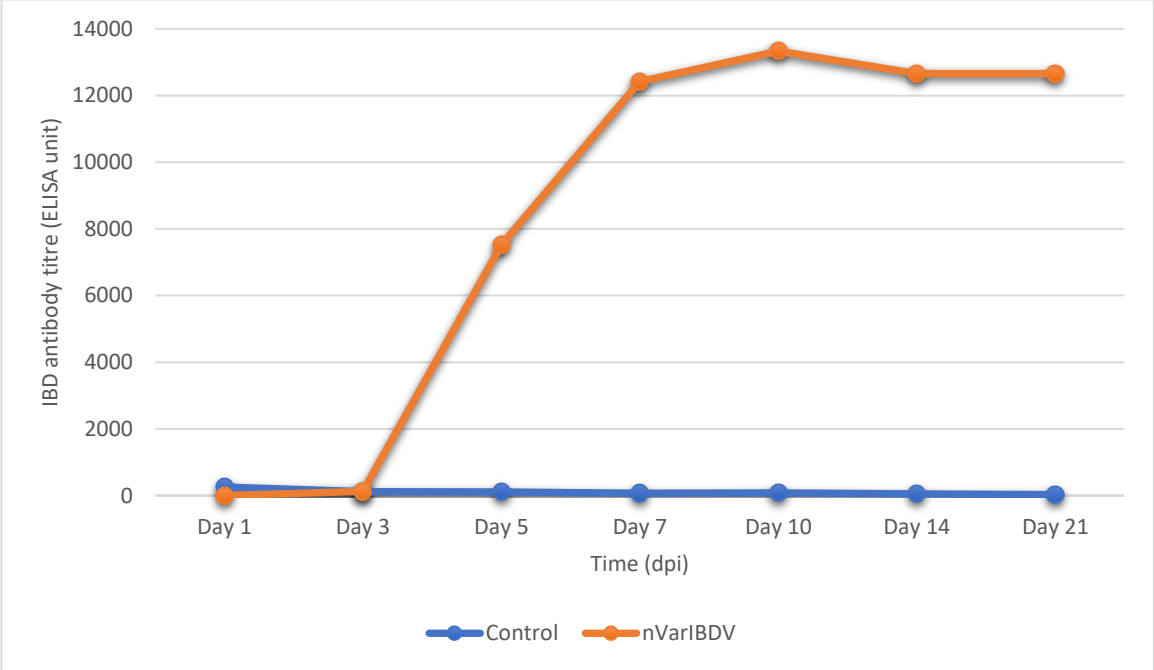

Figure 30: IBD antibody titre of the chickens throughout the study.

4.11 Viral loading (RT-qPCR)

The bursa exhibited the highest viral loads, while the bone marrow displayed the lowest viral loads among these organs. Most organs demonstrated an increasing trend in viral loads from 1 to 3 dpi, reaching their peak at 3 dpi. Subsequently, there was a noticeable decline in viral loads from 3 to 21 dpi, signifying a decreasing trend over time. The virus loads in the bone marrow were initially very low and could not be detected on 14 and 21 dpi. Besides, the thymus has the second-highest virus loads among the organs, followed by the spleen, caecal tonsil, and bone marrow (Figure 27; Appendix 9).

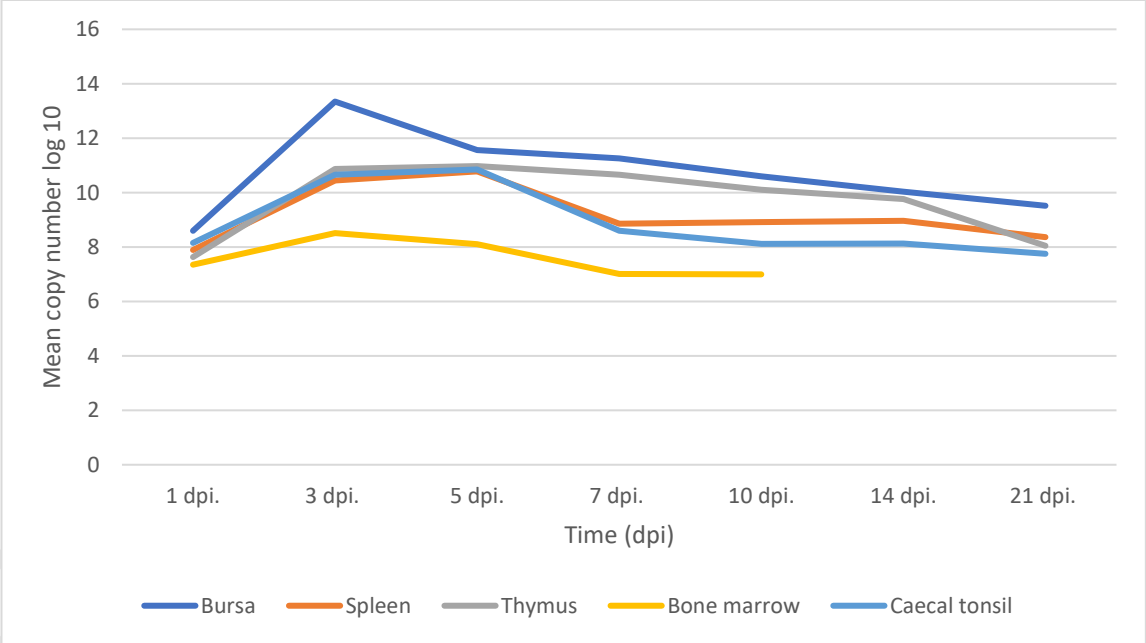

Figure 31: Viral loads in bursa of Fabricius and other organs of the chickens in nVarIBDV group throughout the study.

5.0 Discussion

In this study, the pathogenicity of nVarIBDV was investigated by inoculating nVarIBDV into commercial broiler chickens. Various parameters were assessed, including clinical signs, body weight, bursa weight, bursa-to-body-weight ratio, spleen weight, spleen-to-body-weight ratio, gross lesions, histological lesions, bursal lesion scoring, antibody titer, and viral loads in the organs.

The chickens showed consistent weight gain from 0 to 21 dpi, and no abnormal clinical manifestations were observed. In this study, there were significant differences in body weight between the nVarIBDV group and the control group on 5 dpi, with the body weight of the nVarIBDV group being significantly lower than that of the control group. On the subsequent sampling days, there were no significant differences observed between the nVarIBDV-inoculated chickens and the control group chickens. This suggests that nVarIBDV caused evident adverse effects on the body weight and growth of the chickens that inoculated by nVarIBDV. Despite the absence of clinical signs, the study indicates that nVarIBDV adversely affects chicken growth. This is consistent with the study, nVarIBDV can cause growth retardation in chickens (Fan et al., 2019). This raises concerns, as farmers might overlook the infection, leading to economic losses in the poultry industry due to body weight dropping caused by nVarIBDV.

Over the 21-day trial period, there was a consistent decrease in bursa weight observed in the nVarIBDV-inoculated group from 1 to 21 dpi, while the bursa in the control group chickens displayed a normal increase in weight. The bursa weight of the nVarIBDV group was significantly lower ($p < 0.05$) than that of the control group during 5, 7, 10 and 21 dpi. This implies that nVarIBDV has the potential to severely

impact the bursa, leading to its atrophy. The results also highlighted that nVarIBDV targets the bursa, displaying a tissue tropism, and subsequently causing bursal atrophy. When considering the bursa-to-body-weight ratio, there was a significantly decrease trend in the nVarIBDV-inoculated group from 5 to 21 dpi. This observation suggests that nVarIBDV can affect the bursa, reinforcing its tropism for this specific organ. Additionally, the BBIX index below 0.7 indicates bursal atrophy (Lucio and Hitchner, 1979). From 3 to 21 dpi, the BBIX was lower than 0.7, indicating that bursal atrophy was occurring due to nVarIBDV infection. This is supported by Huang et al. (2023) reported that nVarIBDV caused bursal atrophy with decreased BBIX to below 0.7.

In this study, there were significant differences in spleen weight between the control and nVarIBDV groups on 3 and 5 dpi. The spleen weight of chickens in the nVarIBDV group significantly increased from 3 to 5 dpi, reaching almost twice that of the control group. This substantial increase in spleen weight suggests active nVarIBDV infection from 3 to 5 dpi, resulting in splenomegaly. From 7 to 21 dpi, there were no significant differences in the spleen weights of chickens in both the nVarIBDV and control groups, indicating a stabilization in spleen size as the infection likely reached a different phase. The spleen-to-body-weight ratio of chickens in the nVarIBDV group showed a statistically significant increase compared to the control group on 3 and 5 dpi. This reinforces that there was splenomegaly in response to the nVarIBDV infection.

In terms of gross lesions, the chickens inoculated with nVarIBDV exhibited bursal atrophy consistently throughout the study. This is not consistent with the previous research done by Fan et al. (2019), the bursa exhibits signs of atrophy, hemorrhages and a yellowish appearance due to inflammatory exudation. In this present study, no

other lesions were observed except for bursal atrophy. Again, this reinforces that nVarIBDV can cause bursa atrophy in the nVarIBDV-inoculated chicken, leading to immunosuppression and therefore susceptibility to secondary infection. This may increase the mortality rate of chickens on the farm and cause economic losses in the poultry industry. Additionally, splenomegaly was noted at 3 and 5 dpi. This shows that splenomegaly happened in response to nVarIBDV infection. There were no gross lesions observed in other organs such as the liver, thymus, and muscles. This suggests that nVarIBDV primarily affects the bursa, followed by the spleen, while other organs remain unaffected grossly.

There were no significant histological lesions in the control group (scores of 0 to 1). In the nVarIBDV group, histological changes showed mild to moderate degeneration and necrosis of lymphoid follicles with inflammatory cells infiltration on 3 and 5 dpi. The histological lesions became more severe (scores of 3 to 4) on 7, 10, 14, and 21 dpi, where thickened epithelium, severe depletion of follicular cortices and medullae, connective tissue hyperplasia, infiltration of inflammatory cells within the interstitial spaces, vacuolation, and cyst formation were observed. These findings, along with the increasing bursal lesion score from 3 to 21 dpi in the nVarIBDV-inoculated group, emphasize that the bursa is severely affected by nVarIBDV, causing moderate to severe bursal atrophy (bursal lesion score of 3 to 4), degeneration and necrosis of lymphoid follicles, and fibrosis. This finding corresponds with Huang et al. (2023), Thai et al. (2020) and Fan et al. (2019) who discussed that the histopathological lesions showed lymphocyte depletion, connective tissue hyperplasia, macrophage infiltration in lymphoid follicles, follicular cysts, and infolding of the epithelial lining into damaged follicles. This emphasized that nVarIBDV can cause damage to the bursa, leading to bursal atrophy and immunosuppression.

The IBD antibody titer started to rise from 5 dpi and reached its peak at 10 dpi and remained high until 21dpi. The more pathogenic a virus is towards the host, the higher the antibody titer can be produced by the host. Based on the ELISA results, the antibody titer reached up to 13,000 to 14,000 ELISA units at 10 dpi, which was exceptionally high ELISA titer. This indicates that nVarIBDV is highly pathogenic to commercial broiler chickens, as only then can chickens produce a high immune response.

The RT-qPCR results demonstrated the presence of the virus in all five organs (bursa, spleen, thymus and caecal tonsil throughout the 21 dpi, whilst the bone marrow until 10 dpi.) with the highest viral load observed in the bursa, while the bone marrow had the lowest viral loads among these organs. All five organs showed an increasing trend in viral loads from 1 to 3 dpi, reaching their peak at 3 dpi. Subsequently, there was a decline in viral loads from 3 to 21 dpi, signifying a decreasing trend over time. The viral loads in the bone marrow were very low initially and could not be detected on 14 and 21 dpi. These findings were consistent with the study, there was viral antigen detected in peripheral lymphoid organs such as caecal tonsils, spleen, and thymus, alongside the BF (Dey et al., 2019). The RT-qPCR results proved that nVarIBDV has a high affinity towards the bursa, indicated by the highest viral loads in the bursa. Besides, the results also showed that nVarIBDV infection can persist up to 21 days since the viral loads can be detected until 21 dpi.

6.0 Conclusion

In conclusion, the study demonstrated that nVarIBDV did not cause mortality and did not manifest obvious clinical signs in 3-week-old broiler chickens but have a negative impact to reduce on chicken body weight. The nVarIBDV is pathogenic and caused moderate to severe bursal atrophy (bursal lesion score of 3 to 4), degeneration and necrosis of lymphoid follicles, and fibrosis. Additionally, nVarIBDV caused splenomegaly. There was an immune response with an increasing antibody titer in chickens inoculated with nVarIBDV starting from 5 dpi. The nVarIBDV was detected in the BF, thymus, spleen, caecal tonsil, and bone marrow in nVarIBDV-inoculated chickens, with the bursa having the highest viral loads.

7.0 Recommendations

The recommendations for future studies include the use of a larger sample size to ensure better statistical findings of the results. Additionally, extending the study period would allow further examination of possible effects on the bursa and other organs. Furthermore, the histopathological lesions of other organs such as the spleen, thymus, bone marrow, and caecal tonsil should be examined for a better understanding of the pathogenicity of nVarIBDV.

REFERENCES

Aliyu, H. B., Hamisu, T. M., Hair-Bejo, M., Omar, A. R. and Ideris, A. (2022). Comparative pathogenicity of Malaysian variant and very virulent infectious bursal disease viruses in chickens. *Avian Pathology*, 51 (1): 76-86.

Aliyu, H. B., Hair-Bejo, M., Omar, A. R. and Ideris, A. (2021). Genetic diversity of recent infectious bursal disease viruses isolated from vaccinated poultry flocks in Malaysia. *Frontiers in Veterinary Science*, 8: 643976.

Brandt, M., Yao, K., Liu, M., Heckert, R. A. and Vakharia, V. N. (2001). Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. *Journal of Virology*, 74 (24): 11974-11982.

Chen, Z., Lian, J., Liang, Z., Leng, M., Lin, W. and Chen, F. (2022). Characterization and pathogenicity of infectious bursal disease virus in Southern China. *Poultry Science*, 101 (10): 102018.

Chettle, N., Stuart, J. C. and Wyeth, P. J. (1989). Outbreak of virulent infectious bursal disease in East Anglia. *The Veterinary Record*, 125 (10): 271-272.

Cheville, N. F. (1967). Studies on the pathogenesis of Gumboro disease in the bursa of Fabricius, spleen and thymus of the chicken. *American Journal of Pathology*, 51 (4): 527-551.

Cosgrove, A. S. (1962). An apparently new disease of chickens: avian nephrosis. *Avian Disease*, 6: 385.

Coulibaly, F., Chevalier, C., Gutsche, I., Pous, J., Navaza, J., Bressanelli, S., Delmas, B. and Rey, F. A. (2005). The birnavirus crystal structure reveals structural relationships among icosahedral viruses. *Cell*, 120 (6): 761-772.

Dey S., Pathak D. C., Ramamurthy N., Maity H. K. and Chellappa M. M. (2019). Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. *Veterinary Medicine (Auckland, N. Z.)*, 10: 85-97.

Di, J., Rossini, L., Eterradossi, N., Toquin, M. D. and Gardin, Y. (1999). European-like pathogenic infectious bursal disease viruses in Brazil. *The Veterinary Record,* 145: 203-204.

Fan, L., Wu, T., Hussain, A., Gao, Y., Zeng, X., Wang, Y., Gao, L., Li, K., Wang, Y., Liu, C., Cui, H., Pan, Q., Zhang, Y., Liu, Y., He, H., Wang, X. and Qi, X. (2019). Novel variant strains of infectious bursal disease virus isolated in China.*Veterinary Microbiology,* 230: 212-220.

Fan, L., Wu, T., Wang, Y., Hussain, A., Jiang, N., Gao, L., Li, K., Gao, Y., Liu, C., Cui, H., Pan, Q., Zhang, Y., Wang, X. and Qi, X. (2020). Novel variants of infectious bursal disease virus can severely damage the bursa of Fabricius of immunized chickens. *Veterinary Microbiology*, 240: 108507.

Gilchrist, P. (2005). Involvement of free-flying wild birds in the spread of the viruses of avian influenza, Newcastle disease and infectious bursal disease from poultry products to commercial poultry. *World's Poultry Science Journal*, 61 (2): 198-214.

Hair-Bejo, M., Salina, S., Hafiza, H., and Julaida, S. (2000). In ovo vaccination against infectious bursal disease in broiler chickens. *Journal of Veterinary Malaysia*, 12 (2): 63-69.

Hiraga, M., Nunoya, T., Otaki, Y., Tajima, M., Saito, T. and Nakamura T. (1994). Pathogenesis of highly virulent infectious bursal disease virus infection in intact and bursectomized chickens. *Journal of Veterinary Medical Science*, 56 (6): 1057-1063.

Huang, Y., Shu, G., Huang, C., Han, J., Li, J., Chen, H. and Chen, Z. (2023). Characterization and pathogenicity of a novel variant infectious bursal disease virus in China. *Frontiers in Microbiology*, 13: 1039259.

Kim, I. J., Karaca, K., Pertile, T. L., Erickson, S. A. and Sharma, J. M. (1998). Enhanced expression of cytokine genes in spleen macrophages during acute infection with infectious bursal disease virus in chickens. *Veterinary Immunology and Immunopathology*, 61 (2-4): 331-341.

Lam, K. M. (1997). Morphological evidence of apoptosis in chickens infected with infectious bursal disease virus. *Journal of Comparative Pathology*, 116 (4): 367-377.

Li, K., Niu, X., Jiang, N., Zhang, W., Wang, G., Li, K., Huang, M., Gao, Y., Qi, X. and Wang, X. (2023). Comparative pathogenicity of three strains of infectious bursal disease virus closely related to poultry industry. *Viruses*, 15 (6): 1257.

Lucio, B. and Hitchner, S. B. (1979). Response of susceptible versus immune chicks to killed, live-modified, and wild infectious bursal disease virus vaccines. *Avian Diseases*, 23 (4): 1037-1050.

Mahgoub, H. A., Bailey, M. and Kaiser, P. (2012). An overview of infectious bursal disease. *Archives of Virology*, 157 (11): 2047-2057.

Mwenda, R., Changula, K., Hang'ombe, B. M., Chidumayo, N., Mangani, A. S., Kaira, T., Takada, A., Mweene, A. S. and Simulundu, E. (2018). Characterization of field infectious bursal disease viruses in Zambia: evidence of co-circulation of multiple genotypes with predominance of very virulent strains. *Avian Pathology*, 47 (3): 300-313.

Myint, O., Suwanruengsri, M., Araki, K., Izzati, U. Z., Pornthummawat, A., Nueangphuet, P., Fuke, N., Hirai, T., Jackwood, D. J. and Yamaguchi, R. (2021). The bursa atrophy at 28 days old by the variant infectious bursal

disease virus makes a negative economic impact on broiler farms in Japan. *Avian Pathology*, 50 (1): 6-17.

Orakpoghenor, O., Oladele, S. B. and Abdu, P. A. (2020): Infectious bursal disease: transmission, pathogenesis, pathology and control - an overview. *World's Poultry Science Journal*, 76 (2): 292-303.

Petit, S., Lejal, N., Huet, J. C., and Delmas, B. (2000). Active residues and viral substrate cleavage sites of the protease of the birnavirus infectious pancreatic necrosis virus. *Journal of Virology*, 74 (5): 2057-2066.

Petkov, D. I., Linnemann, E. G., Kapczynski, D. R. and Sellers, H. S. (2009). Identification and characterisation of two distinct bursal B-cell subpopulations following infectious bursal disease virus infection of White Leghorn chickens. *Avian Diseases*, 53 (3): 347-355.

Reed, L. J. and Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology*, 27 (3): 493-497.

Statts, L. E. (2020). Characterization of antigenic variants of infectious bursal disease virus. *University of Delaware, Newark, Delaware, United States.*

Thai, T. N., Jang, I., Kim, H. A., Kim, H. S., Kwon, Y. K. and Kim, H. R. (2021). Characterization of antigenic variant infectious bursal disease virus strains identified in South Korea. *Avian Pathology*, 50 (2): 174-181.

Van, d. B. T. P., Morales, D., Eterradossi, N., Rivallan, G., Toquin, D., Raue, R., Zierenberg, K., Zhang, M. F., Zhu, Y. P., Wang, C. Q., Zheng, H. J., Wang, X., Chen, G. C., Lim, B. L. and Müller, H. (2004). Assessment of genetic, antigenic and pathotypic criteria for the characterization of IBDV strains. *Avian Pathology*, 33 (5): 470-476.

Zhang, W., Wang, X., Gao, Y., and Qi, X. (2022): The over-40-years-epidemic of infectious bursal disease virus in China. *Viruses*, 14 (10): 2253.

Appendices

Appendix 1: Histological lesion score evaluation of the bursa of Fabricius.

Lesion scoring	Description (Thu Zar Than, 1996)
0 (Normal)	Normal or undetectable.
1 (Mild)	Mild degeneration and necrosis especially at the medullary region of lymphoid follicles.
2 (Mild to moderate)	Mild to moderate degeneration and necrosis of lymphoid cells in some lymphoid follicles especially in the medulla. The interstitial connective tissues became oedematous and filled with inflammatory cells.
3 (Moderate)	Moderate necrotised follicles involving both the cortex and medulla. Pyknotic nuclei were scattered in follicles. The interstitial space was obvious and presence of heterophils, macrophages, a few erythrocytes and fibroblast. Epithelial lining was thickened and vacuolated in some area.
4 (Moderate to severe)	Moderate to severe depletion of lymphoid cells in the follicles. Lymphoid cell aggregation found in the cortex of some follicles. Necrotic cells and cysts were present in some follicles especially in the medulla. The interstitial space infiltrated with inflammatory cells and well packed with fibrous connective tissues.
5 (Severe)	Acute or Sub-acute: there were moderate to severe atrophy of the bursal follicles with cellular degeneration and necrosis involving both the cortex and medulla. Follicular cysts with fibrinous exudate and cells debris were frequently observed.

	The interstitial connective tissues were obvious, oedematous and infiltrated with mild to moderate inflammatory cells. The epithelial lining of the bursa was thickened and vacuolated. Chronic: severe follicular atrophy, with cyst formation within the follicles and epithelial lining of the organ. Remarkable infiltration of fibroblast in the interstitial area. Lymphocytes and monocyte infiltration were commonly observed.

Appendix 2: Body weight of chickens throughout the study.

Groups	Body Weight (g)							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV	-	1027.5 ±79.7[a]	1134.0 ± 76.8[a]	1301.8 ± 52.3[a]	1570.5 ± 64.9[a]	1813.5 ± 73.9[a]	2368.0 ± 206.3[a]	2712.8 ± 81.1[a]
Control	824.5 ± 8.8	974 ± 38.2[a]	1113.3 ± 39.4[a]	1427.8 ± 37.5[b]	1321.0 ± 100.6[a]	1863.0 ± 24.8[a]	2111.3 ± 76.2[a]	2970.8 ± 204.2[a]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 3: Bursa of Fabricius weight of chickens throughout the study.

Groups	Bursa of Fabricius weight of chickens (g)							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV	-	1.85 ±0.34[a]	1.45 ± 0.33[a]	0.67 ± 0.14[a]	0.95 ±0.11[a]	0.84 ± 0.12[a]	1.21 ± 0.20[a]	0.81 ± 0.07[a]
Control	1.28 ± 0.01	1.82 ± 0.32[a]	2.27 ± 0.31[a]	2.05 ± 0.21[b]	3.09 ± 0.83[b]	3.76 ± 0.68[b]	3.14 ± 0.60[a]	5.03 ± 0.41[b]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 4: Bursa-to-body-weight ratio of chickens throughout the study.

Groups	Bursa-to-body-weight ratio (10^{-3})							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV	-	1.760 ±0.222[a]	1.318 ± 0.377[a]	0.528 ± 0.127[a]	0.616 ± 0.088[a]	0.458 ± 0.053[a]	0.508 ± 0.076[a]	0.296 ± 0.024[a]
Control	1.554 ± 0.118	1.847 ± 0.267[a]	2.053 ± 0.301[a]	1.423 ± 0.115[b]	2.255 ± 0.418[b]	2.032 ± 0.387[b]	1.468 ± 0.264[b]	1.700 ± 0.130[b]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 5: Spleen weight of chickens throughout the study.

Groups	Spleen weight (g)							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV	-	0.90 ± 0.14[a]	1.47 ± 0.25[a]	2.24 ± 0.14[a]	1.54 ± 0.11[a]	1.92 ± 0.13[a]	1.99 ± 0.26[a]	1.99 ± 0.15[a]
Control	-	0.70 ± 0.12[a]	0.72 ± 0.07[b]	1.11 ± 0.14[b]	1.60 ± 0.20[a]	1.38 ± 0.13[a]	1.75 ± 0.27[a]	2.13 ± 0.16[a]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 6: Spleen-to-body-weight ratio of chickens throughout the study.

Groups	Spleen-to-body-weight ratio (10^{-3})							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV	-	0.873 ± 0.102[a]	1.306 ± 0.239[a]	1.715 ± 0.058[a]	0.985 ± 0.084[a]	1.063 ± 0.086[a]	0.871 ± 0.177[a]	0.729 ± 0.035[a]
Control	-	0.704 ± 0.102[a]	0.645 ± 0.066[b]	0.782 ± 0.113[b]	1.238 ± 0.197[a]	0.744 5 ± 0.081[a]	0.826 ± 0.110[a]	0.725 ± 0.075[a]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 7: Lesion scoring of the bursa of Fabricius throughout the study.

Groups	Lesion scoring of the bursa of Fabricius (0-5)							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV	-	1.25 ± 0.48[a]	4.00 ±0.41[a]	3.00 ± 0.41[a]	4.00 ± 0.41[a]	3.00 ± 0.00[a]	3.00 ± 0.00[a]	4.25 ± 0.25[a]
Control	0.25 ± 0.25	0.75 ± 0.25[a]	0.25 ± 0.25[b]	0.50 ± 0.29[b]	0.75 ± 0.25[b]	0.75 ± 0.25[b]	0.5 ± 0.29[b]	0.25 ± 0.25[b]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 8: IBD antibody titre of chickens throughout the study.

Groups	IBD antibody titre (ELISA Unit)							
	Day post inoculation (dpi)							
	0	1	3	5	7	10	14	21
nVarIBDV		126.5 ± 27.4[a]	135.3 ± 57.8[a]	7519.5 ± 370.7[a]	12414.8 ± 67.0[a]	13332.3 ± 255.2[a]	12658.8 ± 298.5[a]	12654.5 ± 137.7[a]
Control	106.5 ± 35.5	0.0 ± 0.0 [b]	120.3 ± 50.5[a]	106.8 ± 32.0[b]	71.8 ± 17.3[b]	82.0 ± 15.1[b]	54.5 ± 21.1[b]	30.3 ± 11.6[b]

Each value is the mean ± standard error of mean (SEM) of 4 chickens from each group. [a,b] Means with different superscripts within column differed significantly at $p<0.05$.

Appendix 9: Viral loads of various organs in the nVarIBDV-inoculated chickens throughout the study.

Groups	Log_{10} Observed Reaction (Copies/titre)						
	Day post inoculation (dpi)						
	1	3	5	7	10	14	21
Bursa of Fabricius	8.597	13.345	11.566	11.260	10.593	10.034	9.521
Spleen	7.892	10.441	10.780	8.858	8.922	8.961	8.367
Thymus	7.632	10.875	10.975	10.653	10.110	9.767	8.051
Bone marrow	7.351	8.513	8.107	7.007	6.995	Not detected	Not detected
Caecal tonsil	8.152	10.654	10.851	8.602	8.114	8.131	7.752