UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY OF NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN SPECIFIC PATHOGEN FREE CHICKENS

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PATHOGENICITY OF NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN SPECIFIC PATHOGEN FREE CHICKENS

BE TEIN HOCK

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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 Specific Pathogen Free Chickens”, by Be Tein Hock and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 - Final Year Project.

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CONTENTS

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
nVarIBDV	Novel variant infectious bursal disease virus
vvIBDV	Very virulent infectious bursal disease virus
varIBDV	Variant infectious bursal disease virus
caIBDV	Classical infectious bursal disease virus
SPF	Specific pathogen free
VP	Viral protein
GALT	Gut-associated lymphoid tissue
HALT	Head-associated lymphoid tissue
CALT	Conjunctiva-associated lymphoid tissue
RT-qPCR	Real-time quantitative reverse transcription polymerase chain reaction
EID	Embryo infective dose
dpi	Days post inoculation
ELISA	Enzyme-linked immunosorbent assay
CAM	Chorioallantoic membrane
PBS	Phosphate-buffered saline
rpm	Revolutions per minute
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
HE	Hematoxylin & Eosin
BFW	Bursa weight
BFW/BW	Bursa to body weight ratio

SW	Spleen weight
SW/BW	Spleen to body weight ratio
ND	Newcastle disease
AI	Avian influenza

LIST OF APPENDICES

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 - Projek Tahun Akhir.

PATOGENISITI VIRUS PENYAKIT BURSA BERJANGKIT VARIAN NOVEL DALAM AYAM BEBAS PATOGEN SPESIFIK

Oleh

Be Tein Hock

2023

Penyelia: Profesor Dato' Dr Mohd Hair bin Bejo

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Pada tahun 2017, satu jenis baru virus penyakit bursa berjangkit (IBDV) yang dikenali sebagai virus penyakit bursa berjangkit varian novel (nVarIBDV) muncul di China. Strain ini telah menjadi sangat meluas di ladang ayam walaupun telah divaksinasi. Baru-baru ini, kes pertama nVarIBDV dilaporkan di Malaysia pada tahun 2019. Walaupun strain baru ini tidak menyebabkan kematian yang tinggi, ia boleh menyebabkan morbiditi yang ketara dan atrofi bursa yang teruk pada ayam yang terjejas, menyebabkan penekanan imun yang nyata dan seterusnya kerugian ekonomi. Oleh itu, keperluan untuk memahami patogenisiti nVarIBDV di Malaysia adalah sangat penting untuk menyumbang dalam proses diagnostik, pencegahan

dan kawalan penyakit, serta pengeluaran vaksin. Tujuan kajian ini adalah untuk menentukan patogenisiti nVarIBDV dalam ayam bebas patogen spesifik (SPF) dari segi tanda-tanda klinikal, berat badan, parameter bursa dan limpa, lesi kasar dan histologi, titer antibodi, dan beban virus. Sejumlah 60 ayam SPF berumur 21 hari dibahagikan kepada dua kumpulan (Kumpulan A dan Kumpulan B). Ayam dalam Kumpulan A diinokulasikan dengan $10^{6.75}$ EID_{50}/mL nVarIBDV manakala Kumpulan B berperanan sebagai kawalan. Pada 0 hari selepas inokulasi (dpi), empat ekor ayam dari kumpulan B dikorbankan untuk pemeriksaan pra-uji. Empat ekor ayam dari setiap kumpulan dikorbankan pada 1, 3, 5, 7, 10, 14, dan 21 dpi untuk pemeriksaan patogenisiti. Keputusan menunjukkan tanda-tanda klinikal yang tidak normal seperti cirit-birit berair dan bulu yang berderet dalam Kumpulan A. Walau bagaimanapun, tidak terdapat perbezaan yang signifikan ($p>0.05$) antara berat badan kedua-dua kumpulan. Dari segi parameter bursa dan limpa, atrofi bursa dan splenomegali disahkan dalam ayam yang dijangkiti. Dalam bursa, lesi kasar lain seperti bursa berwarna kuning, lipatan bursa yang berkurang, dan konsistensi bursa yang kukuh direkodkan. Lesi histologi bursa menunjukkan atrofi folikel yang parah dan pembentukan cyst besar yang mencapai skor lesi bursa tertinggi iaitu 5. Titer antibodi Kumpulan A adalah lebih tinggi secara signifikan ($p<0.05$) daripada Kumpulan B sejak 5 dpi. Beban virus dalam bursa adalah yang tertinggi manakala ia yang terendah dalam sumsum tulang sepanjang ujian. Oleh itu, penemuan menunjukkan bahawa nVarIBDV menyebabkan jangkitan klinikal yang signifikan dalam ayam SPF dengan tanda-tanda cirit-birit berair dan bulu yang berderet disertai atrofi bursa dan splenomegali yang teruk. Selain itu, ayam yang terinfeksi menunjukkan respons antibodi positif dari 5 dpi dan bursa mempunyai jumlah virus yang tertinggi, tetapi strain ini tidak menyebabkan kematian.

Kata kunci: *Penyakit bursa berjangkit, nVarIBDV, pathogenisiti, ayam bebas patogen spesifik, 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 fulfillment of the course VPD 4999 - Final Year Project.

PATHOGENICITY OF NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN SPECIFIC PATHOGEN FREE CHICKENS

By

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2023

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In 2017, a new strain of infectious bursal disease virus (IBDV) known as novel variant infectious bursal disease virus (nVarIBDV) emerged in China. This strain has become widely prevalent in poultry farms despite vaccination. Recently, the first case of nVarIBDV was reported in Malaysia in 2019. Although this new strain does not typically result in high mortality, it can leads to significant morbidity and severe bursal atrophy in affected chickens causing remarkable immunosuppression and subsequent economic loss. Therefore, the need for understanding the pathogenicity of nVarIBDV in Malaysia is crucially important to contribute in the diagnostic process, disease prevention and control, as well as vaccine production. The objective of this study was to determine the pathogenicity of nVarIBDV in specific pathogen free (SPF)

chickens in terms of clinical signs, body weight, bursa and spleen parameters, gross and histological lesions, antibody titer, and viral loads. A total of 60 21-day-old SPF chickens were divided into two groups (Group A and Group B). Birds in Group A were inoculated with $10^{6.75}$ EID_{50}/mL of nVarIBDV while Group B served as control. At 0 days post inoculation (dpi), four birds from group B were sacrificed for pre-test examination. Four birds from each group were sacrificed at 1, 3, 5, 7, 10, 14, and 21 dpi for pathogenicity examination. The results showed abnormal clinical signs such as watery diarrhea and ruffled feather in Group A. However, there was no significant difference ($p>0.05$) between the body weight of both groups. From the bursa and spleen parameters, bursal atrophy and splenomegaly were verified in the inoculated chickens. In bursa, other gross lesions such as yellowish stained bursa, decreased bursal folds, and firm consistency of bursa were recorded. Histological lesions of bursa showed severe follicular atrophy and large cysts formation which hits the highest bursal lesion score of 5. Antibody titer of group A was significantly higher ($p<0.05$) than group B since 5 dpi. The viral loads in bursa was the highest whereas it was the lowest in bone marrow throughout the trial. Thus, the findings suggested that nVarIBDV causes significant clinical infection in SPF chickens with the signs of watery diarrhea and ruffled feather accompanied by severe bursal atrophy and splenomegaly. Besides, the inoculated chickens showed positive antibody response from 5 dpi and the bursa hosted the highest amount of virus, but this strain does not cause mortality.

Keywords: *Infectious bursal disease, nVarIBDV, pathogenicity, specific pathogen free chicken, atrophy of bursa of Fabricius, IBD antibody, viral loads in organs*

1.0 INTRODUCTION

1.1 Background

Infectious bursal disease (IBD) is an acute and highly contagious viral infection in young chickens caused by IBD virus (IBDV). In 1962, this disease was initially termed as “Avian Nephrosis” due to the severe kidney impairment observed in birds affected by the virus (Swayne *et al.*, 2020). First IBD outbreak was occurred in 1957 in the Gumboro region of Southern Delaware, United States. Hence, this disease is also known as “Gumboro disease” (Cosgrove, 1962). IBDV has tropism towards actively dividing precursor B lymphocytes, primarily located in the bursa of Fabricius. However, other immune organs such as spleen, caecal tonsil, thymus, and bone marrow may also be affected (Aliyu *et al.*, 2022).

IBD brings potential economic impacts in two ways. First, certain strain such as very virulent IBDV (vvIBDV) causes high fatality in chickens older than 3-week-old. Second, IBDV destroy B lymphocytes precursors in the bursa of Fabricius leading to irreversible immunosuppression in the infected chickens (Berg, 2000). The immunosuppressed chickens are more susceptible to secondary infection and vaccination failure (Fan *et al.*, 2019). The acute phase of the disease is characterized by various clinical symptoms such as loss of appetite, lethargy, dehydration, ruffled feathers, diarrhea, prostration, and eventually death. The gross lesions include bursa atrophy, and congestion of the pectoral muscles, hemorrhages might be observed in the thigh, pectoral muscles, and the bursa (Sharma *et al.*, 2000). However, The severity of IBDV infection primarily influenced by the factors such as virus strain, virus load, age and breed of the bird, route of inoculation, and the presence or absence of IBD neutralizing antibodies (Müller *et al.*, 2003).

In 2017, a new virus strain called novel variant IBDV (nVarIBDV) emerged in China, and it is genetically distinct from the original variant IBDV (varIBDV) (Wang *et al*., 2021). This strain has become widely prevalent in chicken farms that have been immunized against IBDV. The first case of nVarIBDV in Malaysia was reported in 2019 (Aliyu *et al.*, 2022). Although this strain of IBDV does not typically results in high mortality, it can leads to significant morbidity and severe bursal atrophy in affected chickens causing significant immunosuppression and subsequent economic loss (Aliyu *et al.*, 2022).

1.2 Justification

1. Emergence of nVarIBDV has potential economic impact in poultry industry due to its high morbidity rate and immunosuppressive effect.
2. Understanding the pathogenicity of nVarIBDV contributing to the diagnostic process, disease prevention and control, and vaccine production.
3. Specific pathogen free (SPF) chickens were used to ensure that there is no influence of IBD antibody on the pathogenicity.

1.3 Hypothesis

Research Hypothesis

nVarIBDV produces significant pathogenicity in SPF chickens in terms of clinical signs, body weight, gross lesions, bursa of Fabricius weight, spleen weight, histological lesions, IBD antibody titer, and viral loads.

Statistical Hypothesis

H_0: There is no significant difference between clinical signs, body weight, gross lesions, bursa of Fabricius weight, spleen weight, and histological lesions, IBD antibody titer, and IBD viral loads in Group A (nVarIBDV inoculated group) and Group B (Control group).

H_A: There is significant difference between clinical signs, body weight, gross lesions, bursa of Fabricius weight, spleen weight, and histological lesions, IBD antibody titer, and IBD viral loads in Group A (nVarIBDV inoculated group) and Group B (Control group).

1.4 Objectives

The overall objective of the study was to determine the pathogenicity of nVarIBDV in specific pathogen free (SPF) chickens.

The specific objectives were;

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

2.0 LITERATURE REVIEW

2.1 Background

IBDV is a double-stranded RNA virus under the avibirnavirus genus and Birnaviridae family. IBDV has a non-enveloped virion with a single shell and icosahedral symmetry. It has a diameter that typically ranges from 55 to 65nm (Müller *et al.*, 2003). The genome of IBDV consists of two segments, segment A and segment B, which are responsible for producing five proteins (VP1 to VP5). Among these proteins, VP2 is the major structural protein that plays a critical role in the pathogenicity of IBDV (Swayne *et al.*, 2020). There are two serotypes of IBDV which are serotype 1 and serotype 2. Only serotype 1 is capable of causing disease in chickens. However, chickens being immunized against serotype 2 does not provide cross-protection against serotype 1 (Swayne *et al.*, 2020). Serotype 1 of IBDV can be categorized into three distinct subtypes based on the virulence, namely classical IBDV (caIBDV), variant IBDV (varIBDV), and very virulent IBDV (vvIBDV) (Aliyu *et al.*, 2021).

2.2 Pathogenesis

Fan *et al.* (2019) suggested that nVarIBDV is prone to horizontal transmission. Under natural condition, the most common routes of transmission for IBDV are oral and ocular routes. In terms of oral route, the virus enters the gut via ingestion of contaminated feed and water. Then, the virus will replicate in the lymphoid cells of the gut-associated lymphoid tissue (GALT) (Sharma *et al.*, 2000). In terms of ocular route, replication of virus occurs in head-associated lymphoid tissues (HALT) which consists of Harderian gland and conjunctiva-associated lymphoid tissue (CALT) (Hair-Bejo, 2010). After the primary replication, the virus will enter blood circulation causing

primary viraemia. The virus will be transported by the phagocytic cells such as macrophages to other tissues, particularly lymphoid organs such as bursa of Fabricius, spleen, caecal tonsil, thymus, and bone marrow for further replication and leading to secondary viraemia (Mahgoub, 2012). Replication of virus in lymphoid organs will leads to immunosuppression due to compromised humoral immunity as the IBDV has tropism towards immature B lymphocytes (Sharma *et al.*, 2000). Therefore, fatality occurs in the flock due to secondary infection such as respiratory and enteric diseases, as well as vaccination failure of important diseases including Newcastle disease and avian influenza (Fan *et al.*, 2021; Wang *et al.*, 2021)

2.3 Clinical signs, gross lesions, and histological lesions

Morbidity rate of nVarIBDV is high, but chickens infected with nVarIBDV does not shows obvious clinical sign except negatively influenced body weight and poor feed conversion rate (Fan *et al.,* 2020). Aliyu *et al.* (2022) reported that SPF chickens infected with Malaysian Variant IBDV (UPM 1432/2019) showed watery whitish diarrhea. In short, nVarIBDV does not leads to serious clinical signs and mortality (Myint *et al.*, 2021).

According to Fan *et al.* (2019), nVarIBDV causes significant gross lesions in the bursa of Fabricius. From 3 to 5 days post-inoculation (dpi), the bursa was atrophied, yellowish stained, haemorrhagic, and inflammatory exudation were observed. Besides, splenomegaly was observed with increased spleen to body weight ratio after 4 dpi (Fan *et al.*, 2019). Thymus atrophy and muscular haemorrhage also occurred in infected chickens (Xu *et al.*, 2020).

Fan *et al.* (2019) reported that prominent histological lesions in the bursa can be observed starting from 1 dpi. The lesions include obviously decreased in lymphocytes

number, infiltration of macrophage, proliferation of fibrous tissue around the follicle, and follicular atrophy. Other than that, Lathasha (2022) also revealed that mild to moderate degeneration and necrosis in the medullary region of lymphoid follicles of bursa with inflammatory cells infiltration were observed in chickens inoculated with pathogenic field strain of nVarIBDV. Few other microscopic lesions recorded by Myint *et al.* (2021) including lymphocyte apoptotic lesions, vacuoles and cystic lesions, and infolding epithelium into the damaged lymphoid follicles indicating significant bursal damage. Aliyu *et al.* (2022) reported that microscopic lesions of spleen can be observed such as lymphoid depletion, congestion, diffused macrophages, regeneration of typical splenic architecture and increased presence of germinal centers.

2.4 Emergence of nVarIBDV in China and around the world

In China, despite effective immunization practices and improved management of vvIBDV, sporadic outbreaks of IBD continue to be reported, and economic losses due to immunosuppression of chickens have gained significant attention since 2017. nVarIBDV were first identified in six provinces of Eastern China. Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR), sequencing, phylogenic analyses, and immunological detection confirmed the antigenic variation of all 50 Chinese variants from American variants in both VP1 and VP2. All Chinese strains isolated exhibited distinct genetic characteristics as evolutionary variants (Fan *et al.*, 2019).

In other countries like Japan, a new antigenic variant strain isolated suggesting high homogeneity to the Chinese variant (Myint *et al.*, 2021). In South Korea, nVarIBDV

was isolated from chickens under passive surveillance and these isolates were similar to those recently emerged in China (Thai *et al.*, 2021).

In Malaysia, molecular detection was carried out between 2017 to 2019 on bursa of flocks of vaccinated commercial broiler in five different states of Malaysia. Among 11 positively tested IBDV variant isolates, UPM1432/2019 and UPM1219/2019 strains were highly identical to the Chinese variant strains based on the sequences and phylogenic analyses (Aliyu *et al.*, 2021).

3.0 MATERIALS AND METHODS

3.1 Experimental design

Sixty 21-day-old SPF chickens were kept in a confinement with controlled lighting and temperature, provided with feed and water *ad libitum*. The chickens were randomly divided into two groups: Group A (nVarIBDV inoculated) and Group B (Control). Group A consists of twenty-eight chickens whereas Group B consist of thirty-two chickens. On 0 dpi, all chickens in Group A were inoculated with 1.0mL of pathogenic field strain nVarIBDV ($10^{6.75}$ EID_{50}/mL) via ocular (0.1mL) and oral (0.9mL) routes, while none for Group B. Clinical signs were monitored at least twice a day and any death and severely sick chickens were sacrificed for sampling throughout the trial. On 0 dpi, four chickens from Group B were sacrificed for pre-test evaluation. On 1, 3, 5, 7, 10, 14, and 21 dpi, four chickens from Group A and four chickens from Group B were selected and sacrificed for data collection each day (Appendix 1). Body weight and serum samples of the selected chickens were collected for detection of IBD antibody titer using Enzyme-linked immunosorbent assay (ELISA) technique prior to sacrifice. Upon necropsy, gross lesions were recorded. Bursa of Fabricius weight and spleen weight were recorded, bursa to body weight ratio and spleen to body weight ratio were calculated. [Bursa to body weight ratio = (Bursa weight / Body weight) X 1000], [Spleen to body weight ratio = (Spleen weight / Body weight) X 1000]. Samples of bursa were collected and fixed in 10% neutral buffered formalin for histological examination and lesion scoring. Samples of bursa, spleen, caecal tonsil, thymus, and bone marrow were also collected into sealed plastic bags for RT-qPCR process.

3.2 nVarIBDV inoculum preparation

Chorioallantoic membrane (CAM) of infected SPF eggs were collected and mixed with Phosphate-buffered saline (PBS) solution in a ratio of 1:2 (CAM:PBS). The mixture was manually homogenized with a mortar and pestle. The homogenized mixture was transferred to a centrifuge tube and centrifuged at 1500 rpm for five minutes. The supernatant containing virus was separated from the debris and filtered with syringe filter to isolate the virus into a sterile tube (Molinet *et al.*, 2023). Before inoculation, titration was done using Reed and Muench method to determine the Embryo Infective Doses per mL (EID_{50}/mL) of the virus (Reed & Muench, 1938). The inoculum was stored at 4^{o}C before use.

3.3 Titration

Ten-fold serial dilutions of nVarIBDV were inoculated into SPF embryonated chicken eggs aged nine to eleven days, with five eggs per dilution administered via the CAM route. The eggs were then incubated at 38^{o}C for a period of seven days. Daily candling of the eggs was performed until 7 dpi, and the mortality rate was recorded. Any mortality within 24 hours post-inoculation were excluded from the calculation, as these deaths were considered as contamination. Contaminated eggs were discarded and could not be used for viral isolation (Soubies *et al.*, 2017). The viral titer were determined by the eggs' morality rate and it was expressed as EID_{50}/mL, using the Reed and Muench formula as shown below (Reed & Muench, 1938).

Reed and Muench formula:

$EID_{50}/mL =$

$$\frac{\text{(\% died at dilution immediately above 50\%) - 50\%}}{\text{(\% died at dilution immediately above 50\%) - (\% died at dilution immediately below 50\%)}}$$

3.4 IBD antibody titer (ELISA)

Blood samples were collected from the chickens at different dpi (0, 1, 3, 5, 7, 10, 14, and 21 dpi). Blood samples were left overnight in the chiller at 2-8°C for serum separation by gravity. Serum obtained were extracted into microtubes and stored in the chiller. Serum were tested for IBD antibody using commercial BioChek IBDV Antibody ELISA Test Kit. The antigen-coated plate first was acclimatised to room temperature (22-27°C) prior to usage. 100 µL of negative and positive controls were dispensed into the wells accordingly, followed by 100µL of 1:500 (v/v) diluted serum samples into respective wells. The plate was covered with lid and incubated at room temperature for 30 minutes. Then, the wells were emptied and washed for 4 times with Wash Solution (350µL per well). After washing, the plate was inverted and tapped firmly on absorbent paper. 100µL of conjugate reagent (anti-chicken IgG labelled with alkaline phosphatase) was then added into each well. The plate was covered with lid and incubated at room temperature for 30 minutes. After 30 minutes, each well was washed again as described previous. 100µL of substrate buffer (diethanolamine buffer with enzyme co-factors) was dispensed into each well. The plate was covered with lid and incubated at room temperature for 15 minutes in the dark. To terminate the reaction, 100µL of stop solution was added into each well. The reaction should be read within 30 minutes. Microtitre plate reader was being used to record the absorbance of

controls and samples at 405nm. The IBD antibody titer was generated using BioCheck 2000 software.

3.5 Viral loads (RT-qPCR)

The pooled samples of bursa of Fabricius, spleen, thymus, bone marrow, and caecal tonsil were collected and fixed in sterile buffer solution (10% neutral buffered formalin) to undergo RT-qPCR. Commercial Kylt® RNA/DNA Purification Kit was being used to purify the RNA of IBDV. The samples were washed out thoroughly by pulse-vortexing and the supernatant contained genetic material (RNA of IBDV) was used. 20mg of tissue per purification was used. Then, 200µL lysis solution, 200µL of sample and 10µL of liquid proteinase K were added to Kylt® Lysis tube accordingly. The mixture was pulse-vortexed for 20 seconds and allowed to spin down for 5 seconds. Then, the mixture was incubated at room temperature for 5 minutes and at 70°C for another 5 minutes. Before the subsequent steps, the sample was cooled down for 2 minutes. For binding, 200µL of 96% ethanol was added. The mixture was pulse-vortexed for 20 seconds and allowed to spin down for 5 seconds. After that, the mixture was transferred to Kylt® Binding column and centrifuged at 10,400 rpm for 1 minute . The collection tube was discarded, and a new collection tube was attached. Then, 500µL of Wash Solution 1 was added to the Kylt® Binding column and centrifuged at 10,400 rpm for 1 minute. The collection tube was discarded, and a new collection tube was attached. Then, 500µL of Wash Solution 2 was added to the Kylt® Binding column and centrifuged at maximum speed (14,000 rpm) for 2 minutes. The collection tube was then discarded, and the elution tube was placed on the column. 100µL of elution buffer was added directly to the membrane without contacting it with the pipette tip. The mixture was incubated at 70°C for 1 minute and centrifuged at maximum speed for 1

minute. The Kylt® Binding column was discarded, and the elution tube was closed. The eluate contains purified RNA and DNA and can immediately be used for RT-qPCR. After extraction of nucleic acid, RT-qPCR assay was being run using commercial SensiFAST™ Probe No-ROX One-Step Kit where reverse transcriptase was used to convert viral RNA to DNA and specific primers were used to amplify the viral DNA. Bio-Rad CFX Opus 96 Real-Time PCR System was used to process and quantify the viral DNA copies in the samples.

3.6 Histopathology and lesion scoring (0 to 5)

Bursa of Fabricius samples were resected from the chicken carcasses during necropsy and fixed with 10% buffered formalin solution for at least 24 hours. Tissues samples were then trimmed cross-sectionally into a thickness of 5mm and processed with an automatic machine (Leica ASP 300) for 24 hours. The samples had undergone a series of dehydration, clearing and impregnation throughout the process. Processed samples were embedded with heated paraffin wax and cooled to solidify. The samples were then trimmed and sectioned into 4µm. After that, fixation on glass slide and hematoxylin and eosin (H&E) staining were performed. The slides were examined under light microscope for histopathological changes of bursa. Bursal lesion scoring was graded based on a scale of 0 to 5; 0 (normal), 1 (mild), 2 (mild to moderate), 3 (moderate), 4 (moderate to severe), 5 (severe) (Thu-Zar, 1996; Appendix 2).

3.7 Statistical analysis

Data collected were analyzed using IBM SPSS Statistical Analysis version 23.0. The confidence interval of this study was 95%, the statistical results were only significant when $p<0.05$.

4.0 RESULTS

4.1 Clinical signs

Throughout the study, there were clinical signs observed in terms of droppings and coat condition. Whitish to yellowish watery droppings were observed in Group A from 3 to 12 dpi (Figure 1). Brownish to reddish watery droppings were observed in Group A on 8, 9 and 18 dpi (Figure 2). Ruffled feathers were observed in Group A from 13 to 21 dpi (Figure 3).

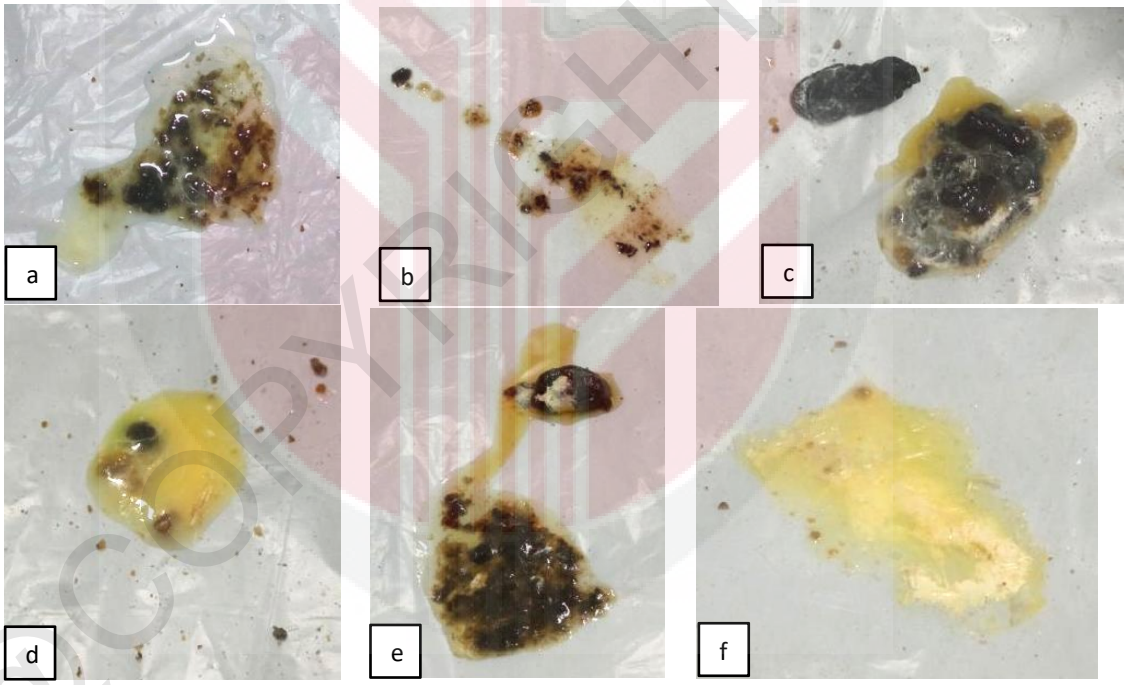

Figure 1: Whitish to yellowish watery droppings in Group A. (a) 3 dpi. (b) 5 dpi. (c) 6 dpi. (d) 7 dpi. (e) 8 dpi. and (f) 9 dpi.

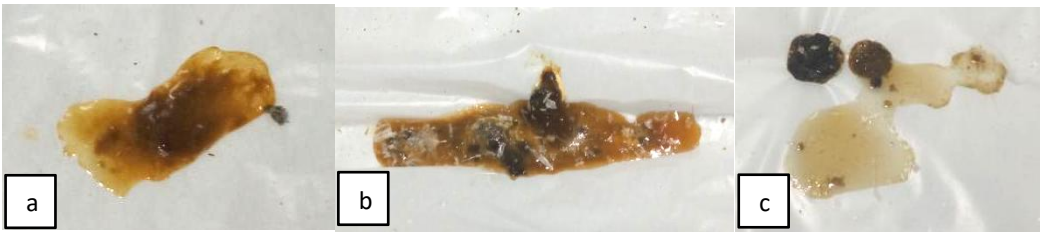

Figure 2: Brownish to reddish watery droppings in Group A. (a) 8 dpi. (b) 9 dpi and (c) 18 dpi.

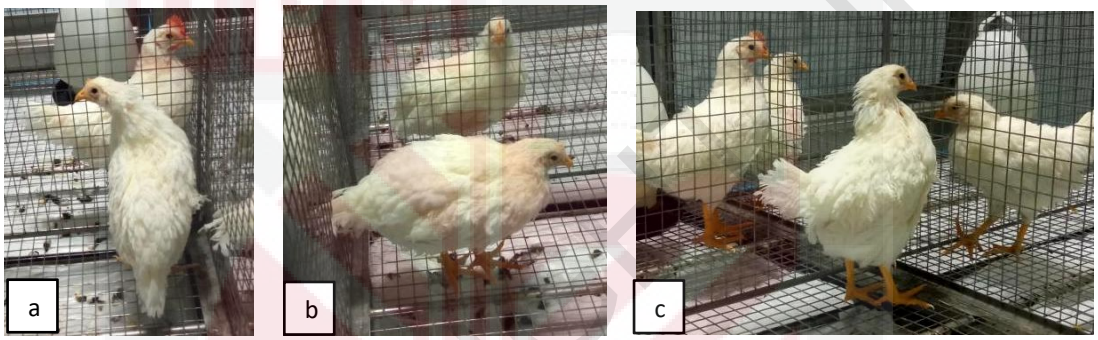

Figure 3: Chickens with ruffled feathers in Group A. (a) 14 dpi. (b) 18 dpi. and (c) 21 dpi.

4.2 Body weight

Throughout the study, there was an overall increase in body weight for both groups. From 0 to 21 dpi, there was no statistically significant difference ($p>0.05$) in body weight between Group A and Group B. (Figure 4; Appendix 3).

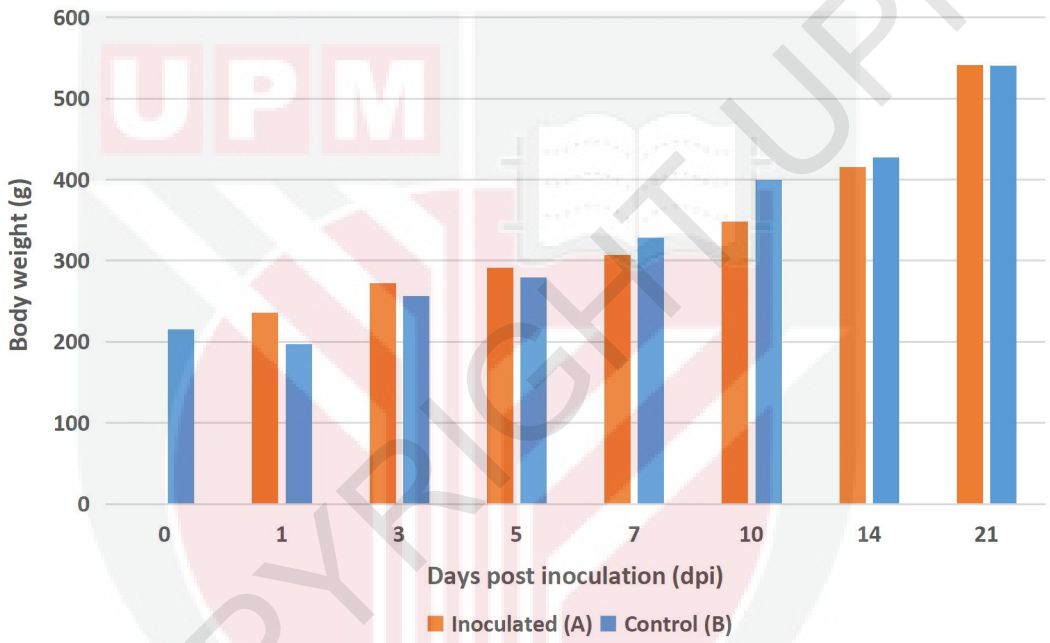

Figure 4: Body weight of chickens throughout the study.

4.3 Bursa weight

The bursa weight of Group B increased steadily from 0 to 21 dpi. On the other hand, the bursa weight of Group A decreased drastically from 1 to 5 dpi and the trend remained stable from 5 to 21 dpi. On day 1 and 3 pi, there was no statistically significant difference ($p>0.05$) in bursa weight between Group A and Group B. From 5 to 21 dpi, bursa weight of Group A was significantly lower ($p<0.05$) than Group B. (Figure 5; Appendix 4).

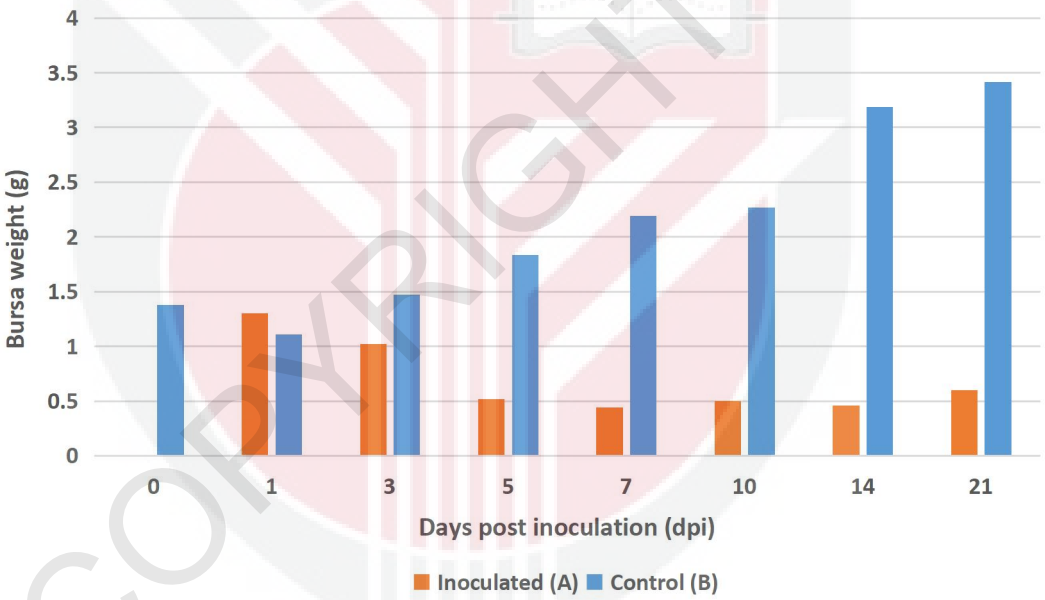

Figure 5: Bursa weight of chickens throughout the study.

4.4 Bursa to body weight ratio

The bursa to body weight ratio of Group A declined steeply from day 1 to day 5 pi and the parameter dropped mildly from 5 to 21 dpi. On 1 dpi, there was no statistically significant difference ($p>0.05$) in bursa to body weight ratio between both groups. From 3 to 21 dpi, the bursa to body weight ratio of Group A is significantly lower ($p<0.05$) than Group B. (Figure 6; Appendix 5).

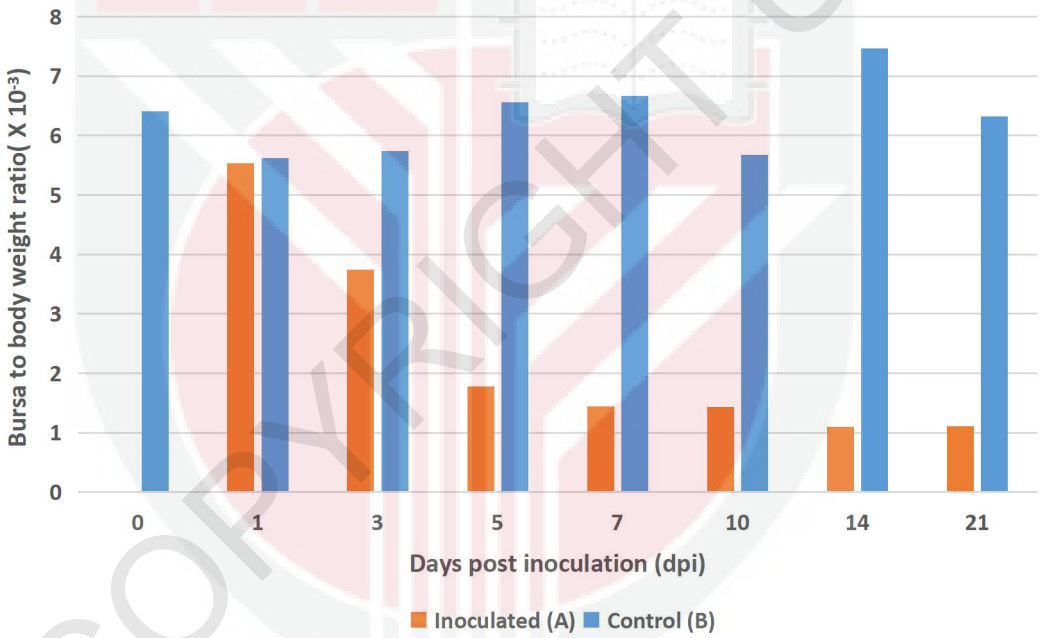

Figure 6: Bursa to body weight ratio of chickens throughout the study.

4.5 Spleen weight

Spleen weight was not collected on 0 dpi. Throughout the trial, there was an overall increase in spleen weight for both groups from day 1 to day 21 pi. From 1 to 21 dpi, there were no statistically significant difference ($p>0.05$) in spleen weight between both groups except on 5 and 7 dpi. On 5 and 7 dpi, the spleen weight of Group A is significantly higher than Group B ($p<0.05$). On 21 dpi, spleen weight of Group A is considerably higher than Group B without statistical significant. (Figure 7; Appendix 6).

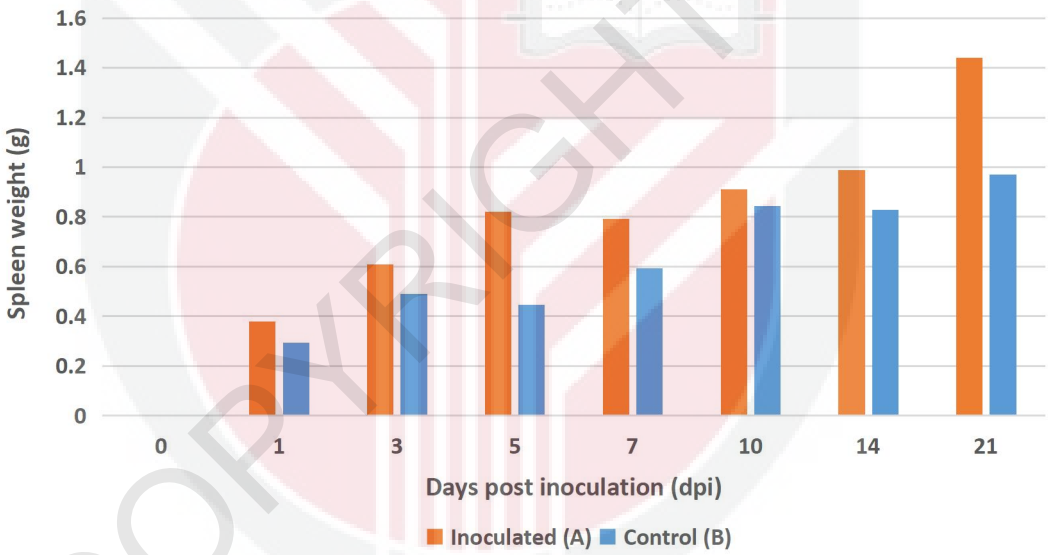

Figure 7: Spleen weight of chickens throughout the study.

4.6 Spleen to body weight ratio

Spleen to body weight ratio was not calculated on 0 dpi as the spleen weight was not collected. From 1 to 21 dpi, there were no statistically significant difference ($p>0.05$) in spleen to body weight ratio between both groups except on 5 and 7 dpi. On 5 and 7 dpi, the spleen to body weight ratio of Group A is significantly higher than Group B ($p<0.05$). On 21 dpi, spleen to body weight ratio of Group A is considerably higher than Group B without statistical significant (Figure 8; Appendix 7).

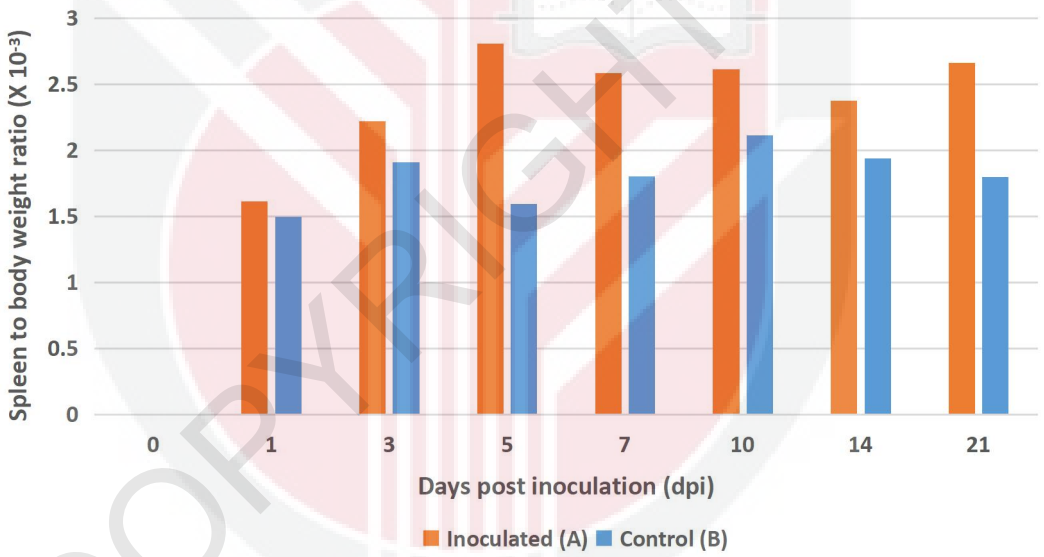

Figure 8: Spleen to body weight ratio of chickens throughout the study.

4.7 Gross lesions

4.7.1 Bursa of Fabricius

There were no abnormal gross lesions observed in the bursa of chickens in Group B from day 0 to day 21 pi (Figures 9 to 16). There were abnormal gross lesions recorded in Group A from 3 to 21 dpi. Bursal atrophy (3, 5, 7, 10, 14, and 21 dpi), yellowish staining of bursa (1, 3, 5, 7, 10, 14, and 21 dpi), decreased bursal folds (3, 5, 7, 10, 14, and 21 dpi), and firm consistency (5, 7, 10, 14, and 21 dpi) were recorded from day 3 to day 21 pi (Figures 10 to 16).

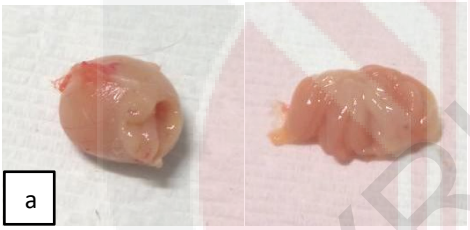

Figure 9: Gross lesion of bursa of chickens on 0 dpi. (a) Group B, normal bursa.

Figure 10: Gross lesion of bursa of chickens on 1 dpi. (a) Group A, yellowish staining of bursa. (b) Group B, normal bursa.

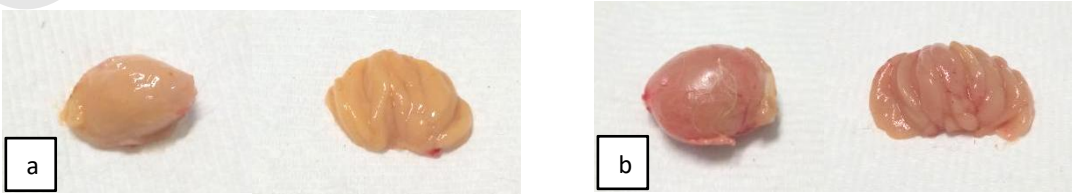

Figure 11: Gross lesion of bursa of chickens on 3 dpi. (a) Group A, bursal atrophy, yellowish staining of bursa and decreased bursal folds. (b) Group B, normal bursa.

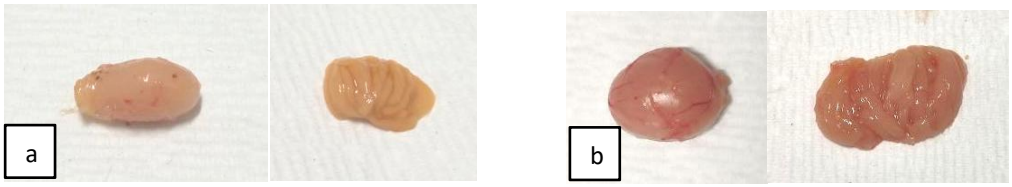

Figure 12: Gross lesion of bursa of chickens on 5 dpi. (a) Group A, bursal atrophy, yellowish staining of bursa, decreased bursal folds and firm consistency. (b) Group B, normal bursa.

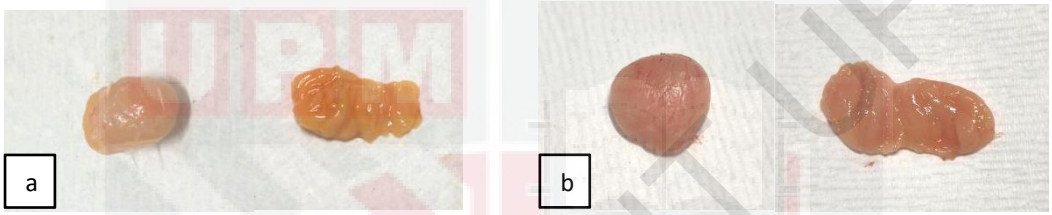

Figure 13: Gross lesion of bursa of chickens on 7 dpi. (a) Group A, bursal atrophy, yellowish staining of bursa, decreased bursal folds and firm consistency. (b) Group B, normal bursa.

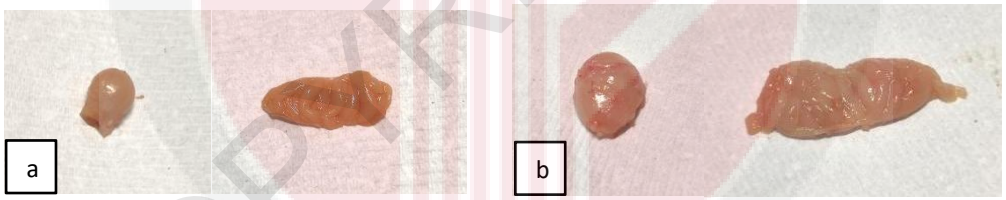

Figure 14: Gross lesion of bursa of chickens on 10 dpi. (a) Group A, bursal atrophy, yellowish staining of bursa, decreased bursal folds and firm consistency. (b) Group B, normal bursa.

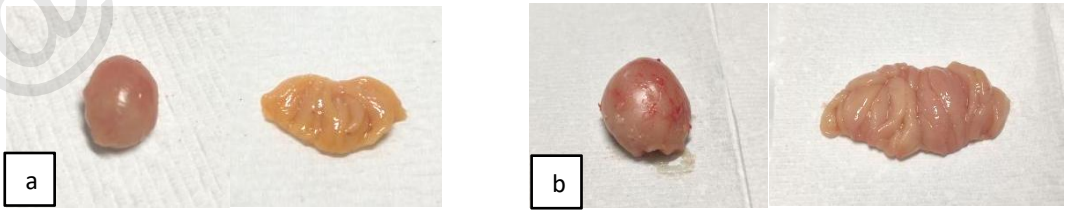

Figure 15: Gross lesion of bursa of chickens on 14 dpi. (a) Group A, bursal atrophy, yellowish staining of bursa, decreased bursal folds and firm consistency. (b) Group B, normal bursa.

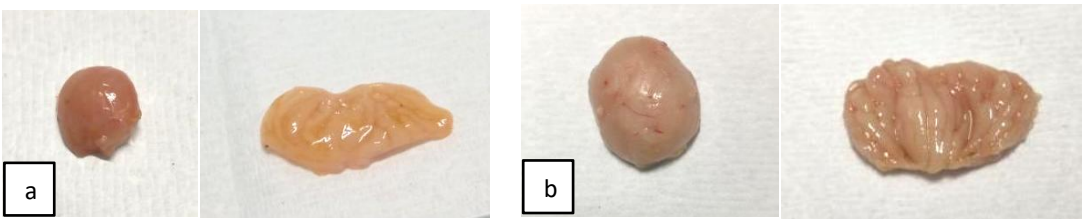

Figure 16: Gross lesion of bursa of chickens on 21 dpi. (a) Group A, bursal atrophy, yellowish staining of bursa, decreased bursal folds and firm consistency. (b) Group B, normal bursa.

4.7.2 Spleen

Throughout the trial, there were no abnormal gross lesion observed in the spleen of Group B from day 0 to day 21 pi (Figures 17 to 24). However, on day 5 and day 7 pi, the spleen in Group A was enlarged as compared to that of Group B (Figures 20 and 21).

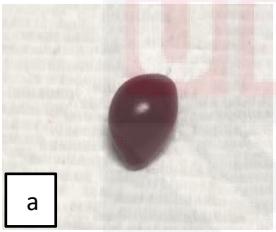

Figure 17: Gross lesion of spleen of chickens on 0 dpi. (a) Group B, normal spleen.

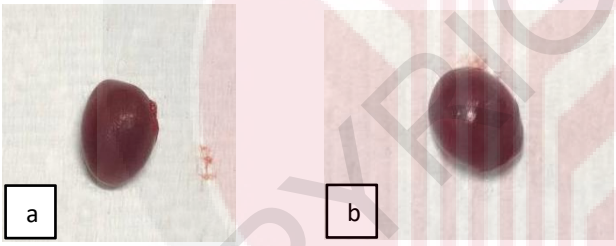

Figure 18: Gross lesion of spleen of chickens on 1 dpi. (a) Group A, normal spleen. (b) Group B, normal spleen.

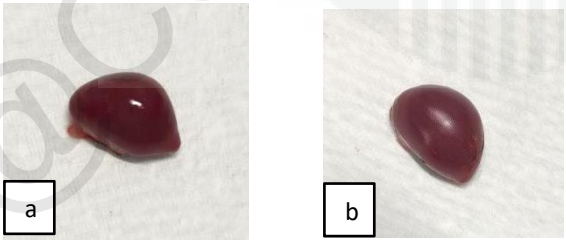

Figure 19: Gross lesion of spleen of chickens on 3 dpi. (a) Group A, normal spleen. (b) Group B, normal spleen.

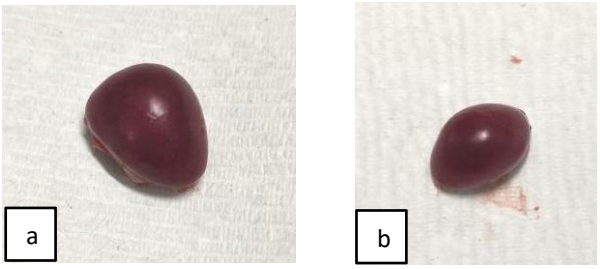

Figure 20: Gross lesion of spleen of chickens on 5 dpi. (a) Group A, splenomegaly. (b) Group B, normal spleen.

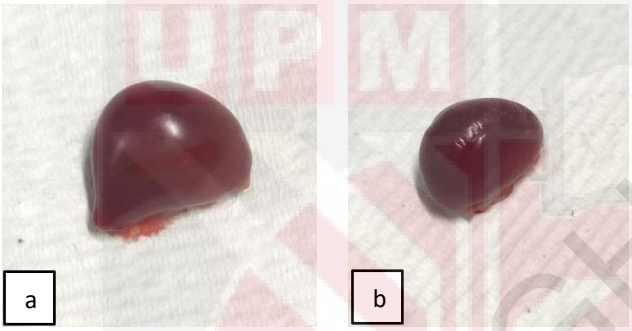

Figure 21: Gross lesion of spleen of chickens on 7 dpi. (a) Group A, splenomegaly. (b) Group B, normal spleen.

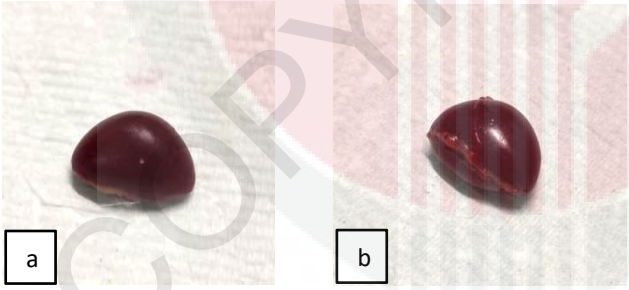

Figure 22: Gross lesion of spleen of chickens on 10 dpi. (a) Group A, normal spleen. (b) Group B, normal spleen.

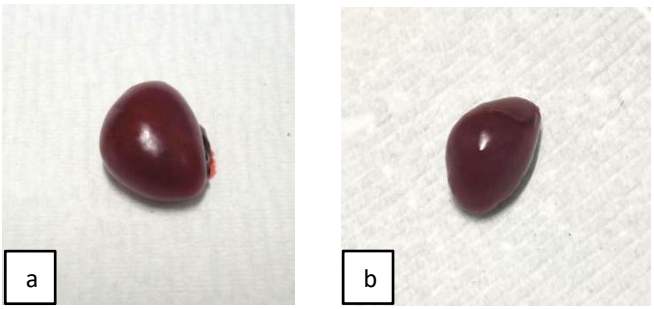

Figure 23: Gross lesion of spleen of chickens on 14 dpi. (a) Group A, normal spleen. (b) Group B, normal spleen.

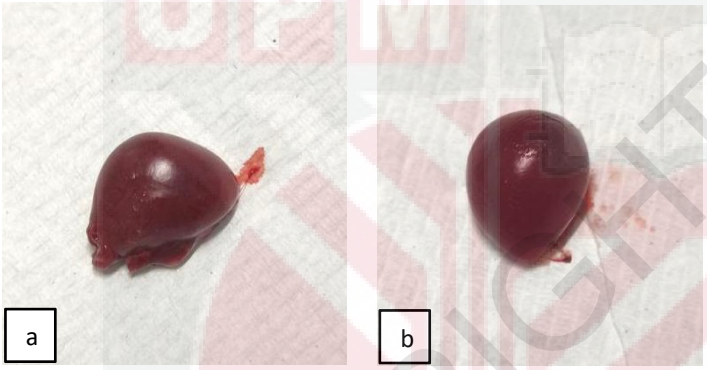

Figure 24: Gross lesion of spleen of chickens on 21 dpi. (a) Group A, normal spleen. (b) Group B, normal spleen.

4.7.3 Thymus

Throughout the trial, there was no abnormal gross lesion observed in the thymus in Group A and Group B from day 1 to day 21 pi (Figures 25 to 31).

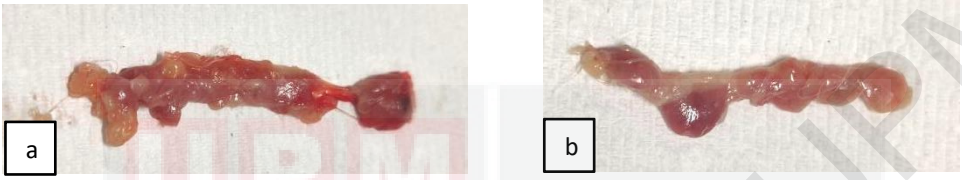

Figure 25: No abnormal gross lesion of thymus of chickens on 1 dpi. (a) Group A. (b) Group B.

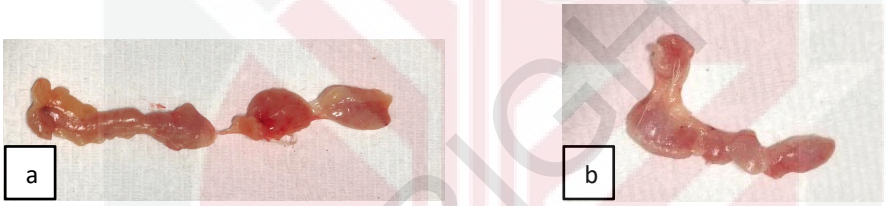

Figure 26: No abnormal gross lesion of thymus of chickens on 3 dpi. (a) Group A. (b) Group B.

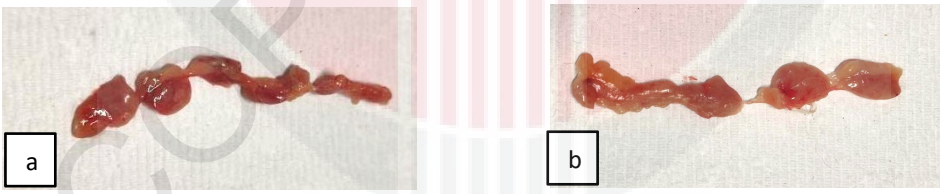

Figure 27: No abnormal gross lesion of thymus of chickens on 5 dpi. (a) Group A. (b) Group B.

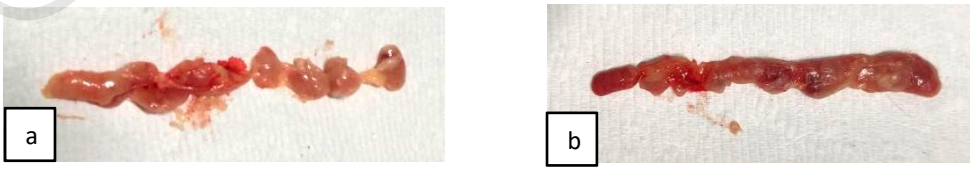

Figure 28: No abnormal gross lesion of thymus of chickens on 7 dpi. (a) Group A. (b) Group B.

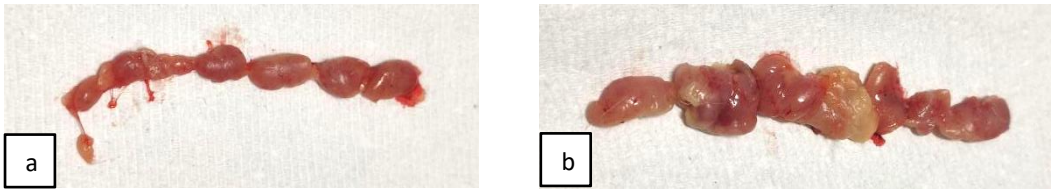

Figure 29: No abnormal gross lesion of thymus of chickens on 10 dpi. (a) Group A. (b) Group B.

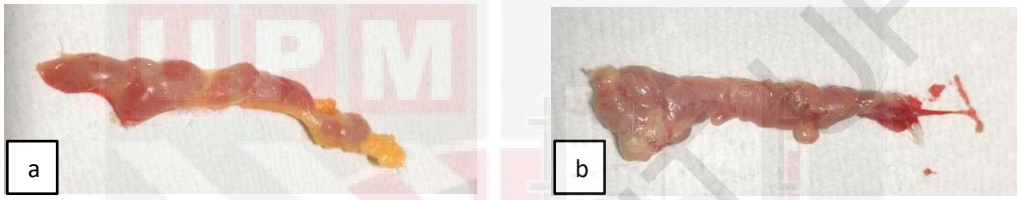

Figure 30: No abnormal gross lesion of thymus of chickens on 14 dpi. (a) Group A. (b) Group B.

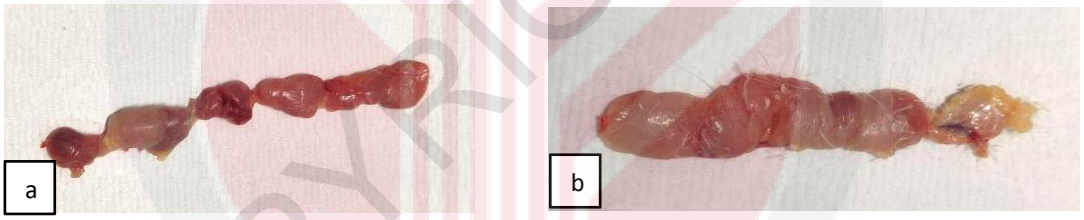

Figure 31: No abnormal gross lesion of thymus of chickens on 21 dpi. (a) Group A. (b) Group B.

4.8 Histological lesions

4.8.1 Bursa of Fabricius

Throughout the trial, there was no abnormal histological lesion observed in Group B from 0 to 21 dpi (Figures 32 to 39). However, there were abnormal histological lesions observed in Group A from day 1 to day 21 pi (Figures 33 to 39).

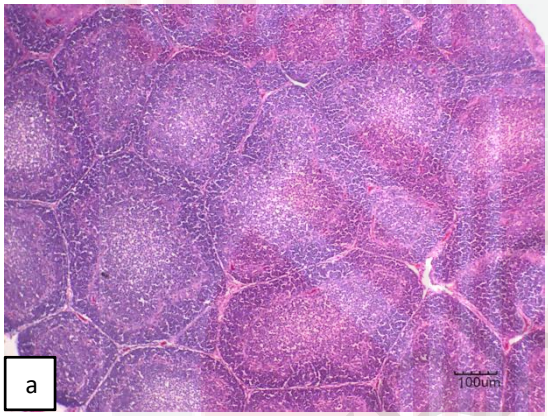

Figure 32: Histopathology of bursa of Fabricius on 0 dpi. (a) Group B (Lesion scoring of 1), intact bursal tissue. HE, 100x, Bar=100µm.

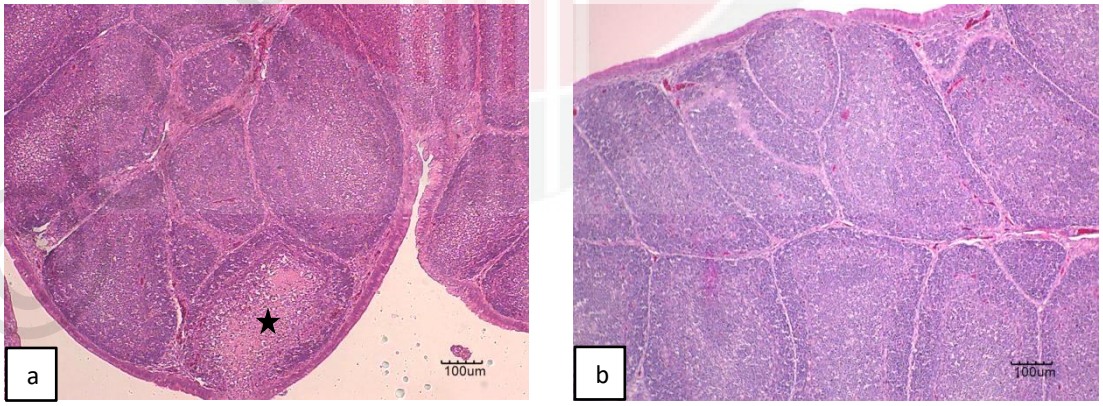

Figure 33: Histopathology of bursa of Fabricius on 1 dpi. (a) Group A (Lesion scoring of 2), mild to moderate lymphoid depletion (star). (b) Group B (Lesion scoring of 0), intact bursal tissue. HE, 100x, Bar=100µm.

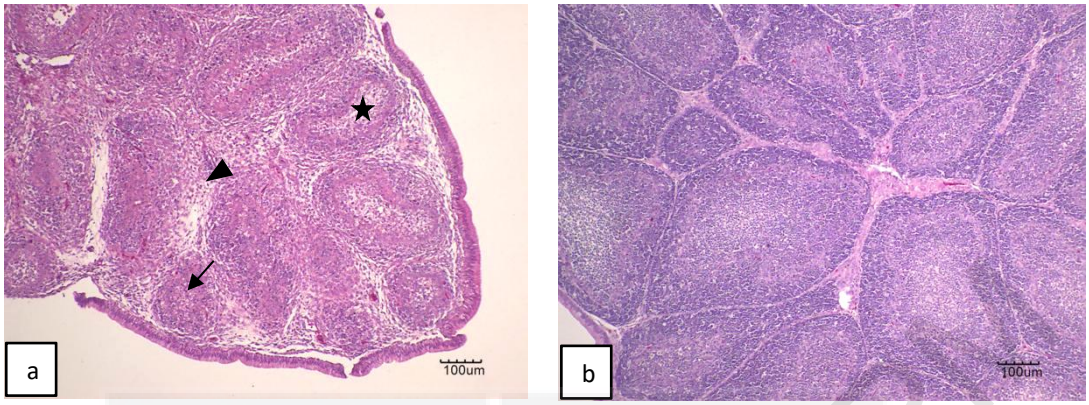

Figure 34: Histopathology of bursa of Fabricius on 3 dpi. (a) Group A (Lesion scoring of 4), moderate to severe lymphoid depletion (star), bursal follicular atrophy (arrow) and oedematous thickened interstitial connective tissue with inflammatory infiltration (arrowhead). (b) Group B (Lesion scoring of 1), intact bursal tissue. HE, 100x, Bar=100µm.

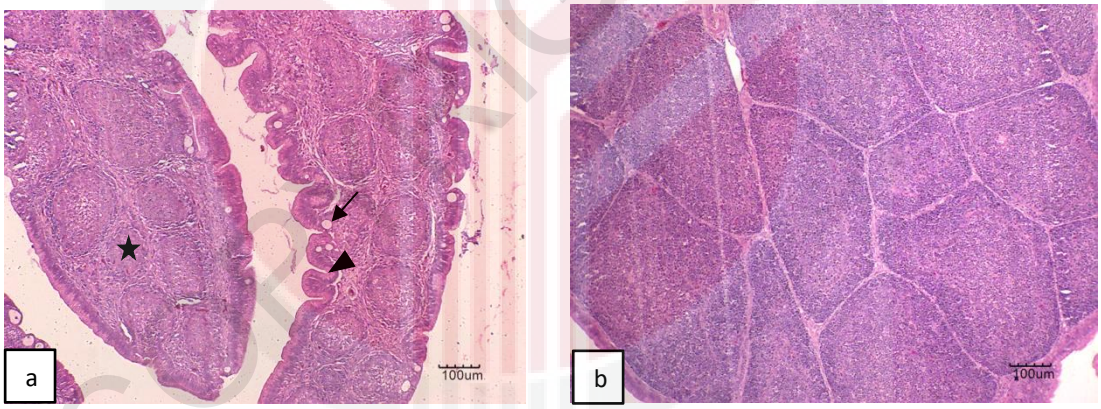

Figure 35: Histopathology of bursa of Fabricius on 5 dpi. (a) Group A (Lesion scoring of 5), bursal follicular atrophy (star), cyst formation at epithelium (arrow) and thickened and corrugated epithelium (arrowhead). (b) Group B (Lesion scoring of 0), intact bursal tissue. HE, 100x, Bar=100µm.

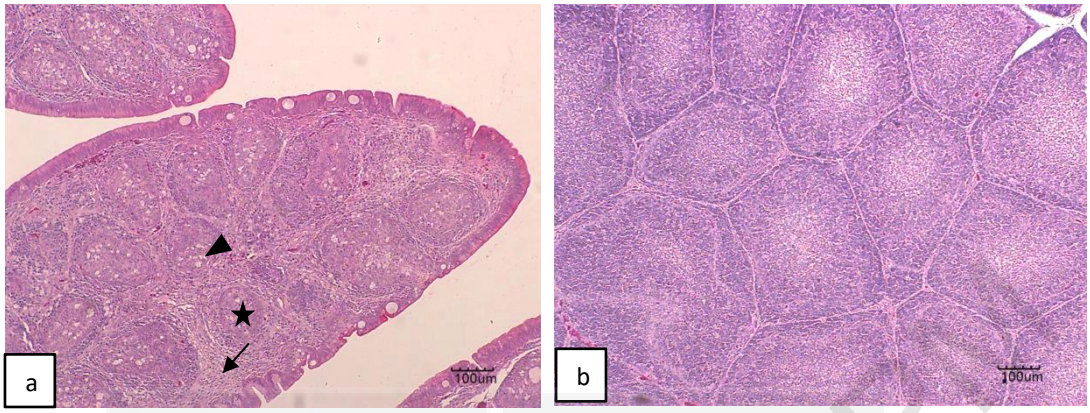

Figure 36: Histopathology of bursa of Fabricius on 7 dpi. (a) Group A (Lesion scoring of 5), bursal follicular atrophy (star), thickened interstitial areas with infiltration of inflammatory cells (arrow) and vacuolation in medullary region (arrowhead). (b) Group B (Lesion scoring of 1), intact bursal tissue. HE, 100x, Bar=100µm.

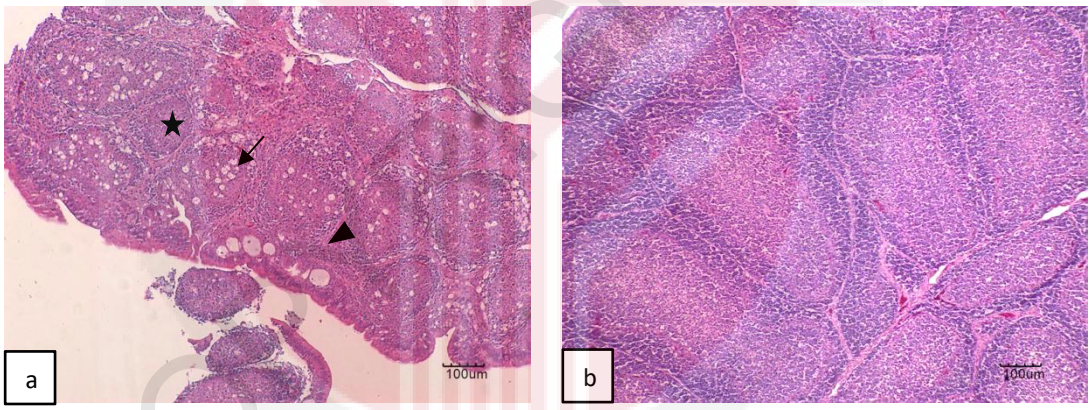

Figure 37: Histopathology of bursa of Fabricius on 10 dpi. (a) Group A (Lesion scoring of 5), bursal follicular atrophy (star), severe vacuolation in medullary region (arrow), and thickened interstitial areas with infiltration of inflammatory cells and proliferation of fibrous tissue (arrowhead). (b) Group B (Lesion scoring of 0), intact bursal tissue. HE, 100x, Bar=100µm.

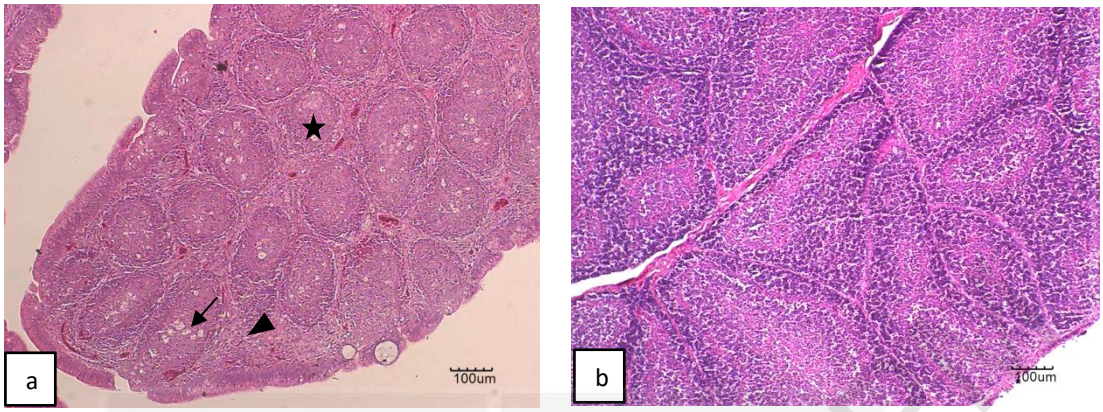

Figure 38: Histopathology of bursa of Fabricius on 14 dpi. (a) Group A (Lesion scoring of 5), bursal follicular atrophy (star), severe vacuolation in medullary region (arrow), and thickened interstitial areas with infiltration of inflammatory cells and proliferation of fibrous tissue (arrowhead). (b) Group B (Lesion scoring of 0), intact bursal tissue. HE, 100x, Bar=100µm.

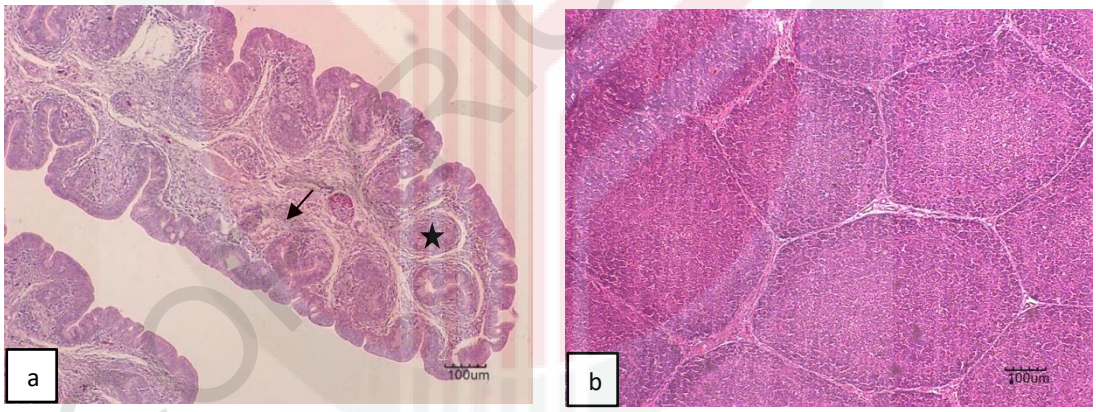

Figure 39: Histopathology of bursa of chickens on 21 dpi. (a) Group A (Lesion scoring of 5), bursal follicular atrophy (star), thickened interstitial areas with infiltration of inflammatory cells and proliferation of fibrous tissue (arrow). (b) Group B (Lesion scoring of 0), intact bursal tissue. HE, 100x, Bar=100µm.

4.8.2 Bursal lesion scoring (0 to 5)

The bursal lesion score of Group B maintained lower than 1 throughout the experiment. The bursal lesion score of Group A climbed sharply from 1 to 3 dpi. The score continue to increase slowly from 3 to 5 dpi and the trend stayed constant until 10 dpi. The score dropped slightly from 10 to 14 dpi and raised back at 21 dpi. On day 1 pi, there was no statistically significant difference ($p>0.05$) in bursal lesion scoring between Group A and Group B. From 3 to 21 dpi, bursal lesion scoring of Group A was significantly higher ($p<0.05$) than Group B (Figure 40; Appendix 8).

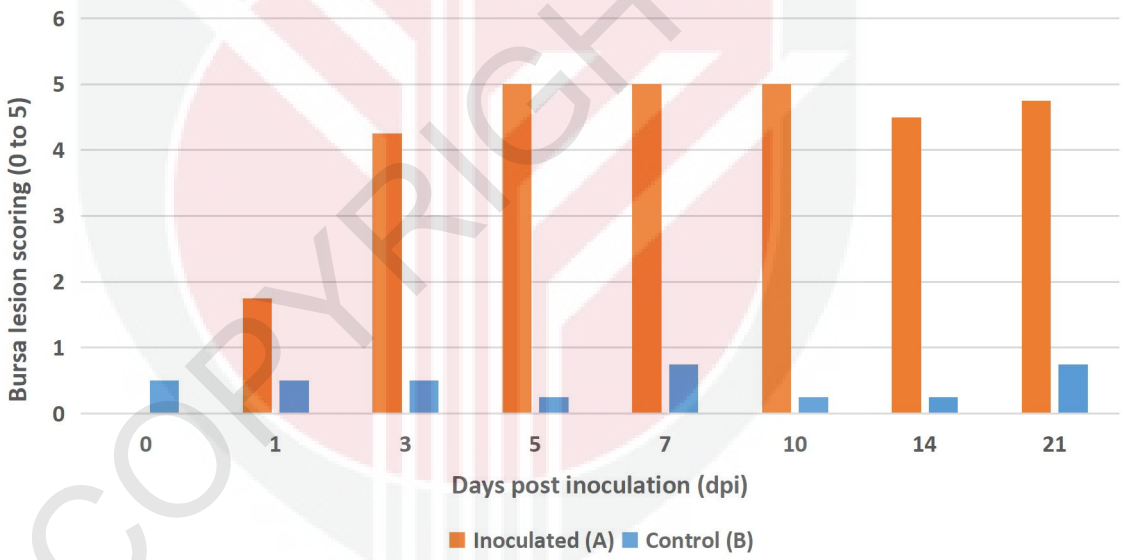

Figure 40: Bursal lesion scoring of chickens throughout the study.

4.9 IBD antibody titer (ELISA)

IBD antibody started to appear on 5 dpi. IBD antibody titer increased rapidly from 5 to 7 dpi and slowly reduced from 7 dpi to 21 dpi . From 1 to 5 dpi, there were no statistically significant difference ($p>0.05$) in IBD antibody titer between Group A and Group B. From 7 to 21 dpi, IBD antibody titer of Group A was significant higher ($p<0.05$) than Group B (Figure 41; Appendix 9).

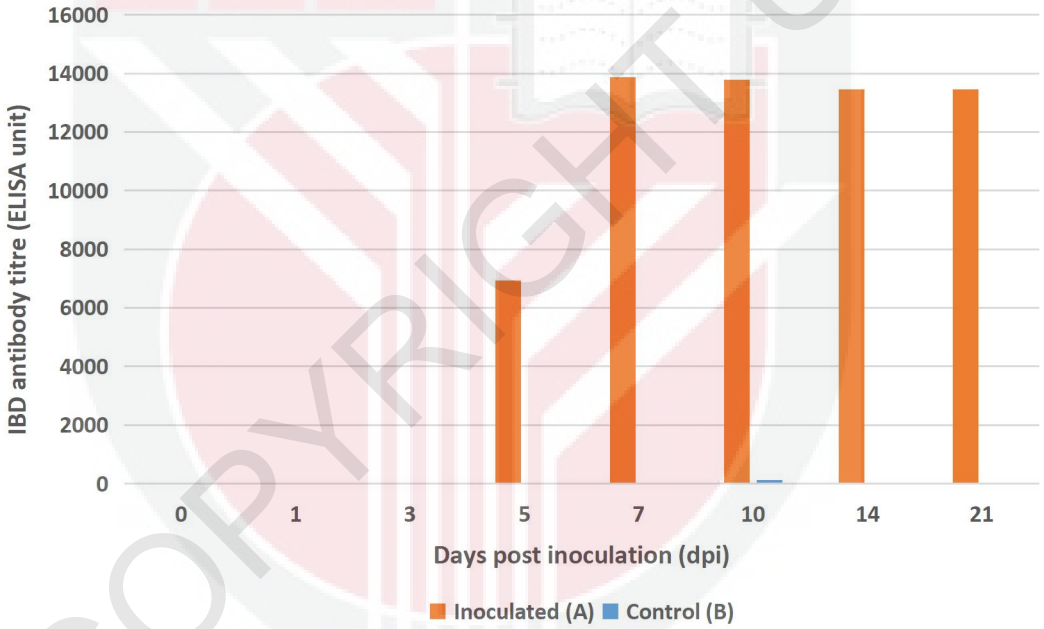

Figure 41: IBD antibody titer of the chickens throughout the study.

4.10 Viral loading (RT-qPCR)

Bursa hosted the highest viral loads of nVarIBDV while the bone marrow was the lowest. Viral loads in bursa and thymus hit the peak at 3 dpi and decrease gradually. Besides, the viral loads in spleen and bone marrow reached the peak at 5 dpi and caecal tonsil has the highest viral loads at 7 dpi. All organs reached the peak viral loads between 3 to 7 dpi. Viral loads in bone marrow was not detected after 7 dpi (Figure 42; Appendix 10).

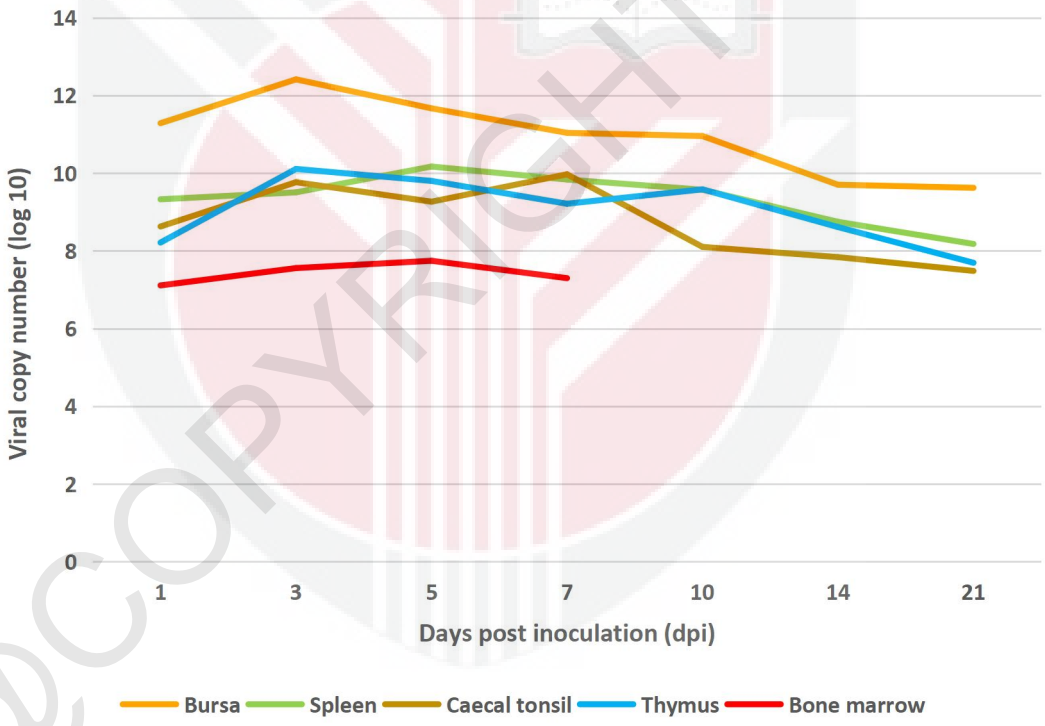

Figure 42: Viral loads in each organs of the chickens in Group A throughout the study.

5.0 DISCUSSION

The pathogenicity of nVarIBDV was evaluated by 11 parameters, which were 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, IBD antibody titer, and viral loading.

Throughout the study, no mortality was recorded, but there were some abnormal clinical signs being observed in the inoculated chickens which are ruffled feathers and whitish to yellowish or brownish to reddish watery diarrhea. Aliyu *et al.* (2022) reported that infected chickens showed watery whitish diarrhea, and no fatality was recorded. Huang *et al.* (2023) and Fan *et al.* (2019) concluded that there were no obvious gross clinical signs or death being observed in the infected chickens. Thus, the current findings correspond with the previous studies, nVarIBDV does not cause mortality but it leads to minor clinical signs such as watery diarrhea and ruffled feathers in SPF chickens.

In terms of body weight, no significant difference was recorded between Group A and Group B. Besides, body weight of both control and infected groups increased steadily from day 0 to day 21 pi. Huang *et al.* (2023) reported that body weight (BW) of the infected chickens were negatively impacted. Fan *et al.* (2020) described that nVarIBDV induced weight loss of infected chickens. Therefore, the results obtained were inconsistent to the previous studies, nVarIBDV did not interfere with the growth performance of the inoculated chickens. This is because SPF layer chickens were used in this trial. The growth rate of SPF chickens is relatively low as compared to commercial broiler.

Along the trial, there was an overall increase in bursa weight (BFW) of Group B whereas the bursa weight of Group A decreased from 1 to 21 dpi. The bursa weight of Group A is significantly lower than Group B starting from 5 to 21 dpi. According to

previous researches in China, Japan, South Korea, and Malaysia, severe bursal atrophy was observed in SPF chickens as well as commercial broilers (Aliyu *et al.*, 2022; Myint *et al.*, 2021; Thai *et al.*, 2021; Fan *et al.*, 2019). Hence, The current data collected corresponds with the past results. Based on the bursa weight data, it can be concluded that nVarIBDV only causes significant bursal damage beginning from 5 dpi that leads to bursal atrophy and the impact persists until 21 dpi.

Other than BFW, bursa to body weight ratio (BFW/BW) is a more precise parameter to assess the bursal atrophy. This is because the bursa weight of the chickens is expected to increase along the trial as the body weight increases. Throughout the experiment, there was an overall decrease in BFW/BW of chickens in Group A from 1 to 21 dpi. BFW/BW of Group A is significantly lower than Group B from 3 to 21 dpi. Thus, a conclusion can be made that nVarIBDV infects the bursa and causing it to be atrophied at 3 dpi. Bursal atrophy indicating that there is bursal damage. The infected chickens tend to be immunosuppressed and more susceptible to secondary infection as well as vaccination failure. This is supported by Fan *et al.* (2020 & 2019) reported that nVarIBDV decreases the efficacy of Newcastle Disease (ND) and Avian Influenza (AI) vaccination.

There were no abnormal gross lesions observed in the bursa of chickens in Group B from day 0 to day 21 pi. On the other hand, severe bursal atrophy, yellowish staining of bursa, decreased bursal folds, and firm consistency of the bursa were recorded from day 3 to day 21 pi. Gross lesions of bursa indicating that the bursa has been damaged by the nVarIBDV that leads to immunosuppression in chickens.

Throughout the 21-days experiment period, significant difference in spleen weight (SW) between chickens in inoculated and control groups were only been observed on 5 and 7 dpi. SW of the inoculated group is significantly higher ($p<0.05$) than the control group

on 5 and 7 dpi. Therefore, this shows that nVarIBDV causes splenomegaly in SPF chickens only within period of 5 to 7 dpi, recovery occurred after 7 dpi until 21 dpi.

Another parameter that is more accurate than SW to evaluate splenomegaly is spleen to body weight ratio (SW/BW). Results obtained were similar to SW. SW/BW of the inoculated group was significantly higher ($p<0.05$) than the control group within the period of 5 to 7 dpi. This finding consistent with the previous studies done by Aliyu *et al.* (2022) who stated that the infected SPF chickens showed a significantly increased value in SW/BW compared to the control group at 4 and 5 dpi. Hence, it is logical to speculate that nVarIBDV only causes significant splenic damage within 5 to 7 dpi, the immunity of the chicken is able to overcome the virus and initiate a regeneration process of spleen.

As for the gross lesion of spleen, splenomegaly can be observed in Group A at 5 to 7 dpi as compared to that of Group B. This finding corresponds with Fan *et al.* (2019) who discussed that nVarIBDV causes severe congestion and swelling of the spleen.

The inoculated SPF chickens started to produce IBD antibody at 5 dpi. The antibody titer then increased rapidly from 5 to 7 dpi and slowly decreased. In addition, the antibody titer of the inoculated chickens were extremely high which was ranging between 13,000 to 14,000 ELISA units within 7 to 21 dpi period. This was uncommon as according to the previous research done by Aliyu *et al.* (2019), the antibody titer of nVarIBDV infected SPF chickens was only ranging between 5,000 to 10,000 ELISA units. High antibody titer indicating that nVarIBDV is highly pathogenic to SPF chickens.

Bursa hosted the highest viral loads of nVarIBDV while the bone marrow was the lowest. From this finding, we can deduce that bursa is the primary target of nVarIBDV while bone marrow is not the main target. Viral loads in bursa and thymus hit the peak at 3 dpi and started to decrease gradually. Besides, the viral loads in spleen and bone

marrow reached the peak at 5 dpi and caecal tonsil had the highest viral loads at 7dpi. nVarIBDV persists long in all five organs except bone marrow indicating that the infection period is long. This result is supported by Aliyu *et al.* (2019) reported that nVarIBDV is still detected in bursa and spleen at 21 dpi. All organs reached the peak viral loads between 3 to 7 dpi. Viral loads in bone marrow was not detected after 7 dpi.

6.0 CONCLUSION

In conclusion, this study demonstrated that nVarIBDV is highly pathogenic to SPF chickens. nVarIBDV causes watery diarrhea and ruffled feathers but no other obvious clinical signs observed, nVarIBDV causes severe bursal atrophy, yellowish stained bursa, decreased bursal folds, and firm consistency of bursa grossly. nVarIBDV causes severe follicular degeneration, necrosis, fibrosis, inflammation, and atrophy in the bursa microscopically with bursal lesion scoring of 4 to 5. nVarIBDV causes splenomegaly. The inoculated chickens started to produce antibody from 5 dpi. Bursa hosted the highest viral loads and peaked at 3 dpi.

7.0 RECOMMENDATIONS

In future studies, it is recommended to increase the sample size for better statistical findings. Besides, the experiment period should be extended to observe for immunosuppressive effect of nVarIBDV. Lastly, microscopic lesions of other lymphoid organs such as spleen, thymus, bone marrow, and caecal tonsil should be examined for a better understanding of the pathogenicity of nVarIBDV.

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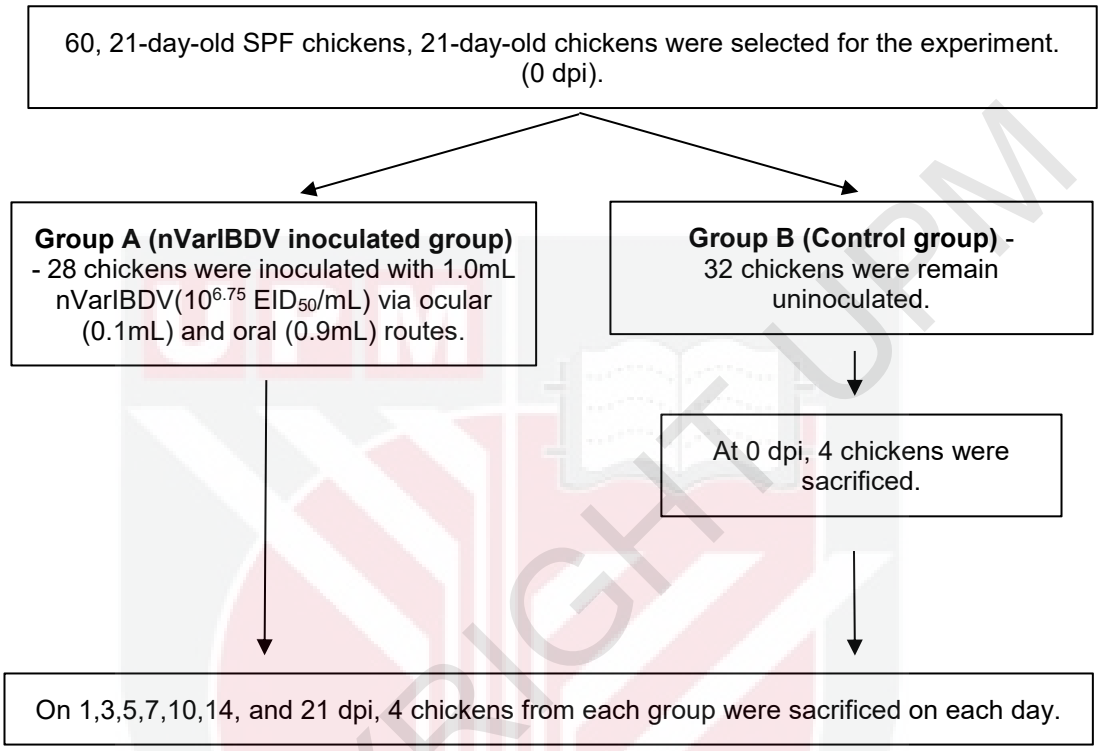
APPENDICES

APPENDIX 1: Experimental design



APPENDIX 2

Histological lesion scoring of the bursa of Fabricius (Thu-Zar, 1996).

Lesion score	Description
0	Normal or undetectable.
1	Mild degeneration and necrosis especially at the medullary region of lymphoid follicles.
2	Mild to moderate degeneration and necrosis especially at the medullary region of lymphoid follicles. Oedematous interstitial connective tissues and infiltration of inflammatory cells.
3	Moderate necrotised follicles involving both cortex and medulla. Presence of pyknotic nuclei in the follicles with obvious interstitial space filled with heterophils, macrophages and fibroblast. Epithelial lining was thickened and vacuolated in some area.
4	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. The intra and extra follicular areas might be hyperaemic and haemorrhagic. Epithelium was thickened, corrugated and vacuolated in some areas.

5	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 3: Body weight of chickens throughout the trial.

Groups	Body weight (g)							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	235.5 ± 15.9[a]	272.5 ± 10.1[a]	291.5 ± 13.0[a]	307.0 ± 12.4[a]	348.5 ± 10.3[a]	415.5 ± 62.9[a]	541.0 ± 41.0[a]
B (Control)	215.3 ± 10.1	197.0 ± 18.6[a]	256.5 ± 8.7[a]	279.5 ± 13.1[a]	328.5 ± 17.9[a]	399.8 ± 25.5[a]	427.0 ± 31.7[a]	540.3 ± 23.1[a]

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

APPENDIX 4: Bursa weight of chickens throughout the trial.

Groups	Bursa weight (g)							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	1.30 ± 0.09[a]	1.02 ± 0.10[a]	0.52 ± 0.07[a]	0.45 ± 0.03[a]	0.50 ± 0.07[a]	0.46 ± 0.94[a]	0.60 ± 0.13[a]
B (Control)	1.38 ± 0.10	1.11 ± 0.17[a]	1.47 ± 0.22[a]	1.83 ± 0.19[b]	2.19 ± 0.46[b]	2.27 ± 0.39[b]	3.19 ± 0.90[b]	3.42 ± 0.49[b]

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

APPENDIX 5: Bursa to body weight ratio of chickens throughout the trial.

Groups	Bursa to body weight ratio ($x10^{-3}$)							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	5.565 ± 0.343[a]	3.738 ± 0.308[a]	1.781 ± 0.232[a]	1.447 ± 0.049[a]	1.426 ± 0.169[a]	1.079 ± 0.063[a]	1.086 ± 0.171[a]
B (Control)	6.400 ± 0.254	5.561 ± 0.547[a]	5.683 ± 0.674[b]	6.546 ± 0.554[b]	6.608 ± 1.217[b]	5.574 ± 0.704[b]	7.236 ± 1.511[b]	6.425 ± 1.131[b]

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

APPENDIX 6: Spleen weight of chickens throughout the trial.

Groups	Spleen Weight (g)							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	0.38 ± 0.06^{a}	0.61 ± 0.05^{a}	0.82 ± 0.10^{a}	0.79 ± 0.08^{a}	0.91 ± 0.07^{a}	0.99 ± 0.14^{a}	1.44 ± 0.20^{a}
B (Control)	-	0.30 ± 0.05^{a}	0.49 ± 0.07^{a}	0.45 ± 0.06^{b}	0.59 ± 0.02^{b}	0.85 ± 0.106^{a}	0.83 ± 0.09^{a}	0.97 ± 0.08^{a}

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

APPENDIX 7: Spleen to body weight ratio of chickens throughout the trial.

Groups	Spleen to Body Weight Ratio ($x10^{-3}$)							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	1.593 ± 0.237[a]	2.234 ± 0.194[a]	2.774 ± 0.249[a]	2.568 ± 0.167[a]	2.603 ± 0.164[a]	2.421 ± 0.224[a]	2.661 ± 0.344[a]
B (Control)	-	1.468 ± 0.143[a]	1.894 ± 0.233[a]	1.586 ± 0.182[b]	1.823 ± 0.132[b]	2.120 ± 0.249[a]	1.943 ± 0.178[a]	1.797 ± 0.143[a]

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

APPENDIX 8: Lesion scoring of the bursa of Fabricius throughout the trial.

Groups	Bursal Lesion Scoring							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	1.75 ± 0.48[a]	4.25 ± 0.25[a]	5.00 ± 0.00[a]	5.00 ± 0.00[a]	5.00 ± 0.00[a]	4.50 ± 0.29[a]	4.75 ± 0.25[a]
B (Control)	0.50 ± 0.29[a]	0.50 ± 0.29[a]	0.50 ± 0.29[b]	0.25 ± 0.25[b]	0.75 ± 0.25[b]	0.25 ± 0.25[b]	0.25 ± 0.25[b]	0.75 ± 0.25[b]

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

APPENDIX 9: IBD antibody titer of chickens throughout the trial.

Groups	IBD antibody titer (ELISA unit)							
	Days post inoculation (dpi)							
	0	**1**	**3**	**5**	**7**	**10**	**14**	**21**
A (Inoculated)	-	9.8 ± 8.8[a]	45.3 ± 3.3[a]	6920.8 ± 484.6[a]	13869.0 ± 170.5[a]	13786.0 ± 59.2[a]	13457.5 ± 332.3[a]	13443.5 ± 222.3[a]
B (Control)	1.0 ± 0.0[a]	8.5 ± 7.5[a]	1.0 ± 0.0[b]	21.0 ± 20.0[b]	7.8 ± 4.0[b]	128.8 ± 125.8[b]	10.2 ± 8.6[b]	8.0 ± 4.4[b]

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

APPENDIX 10: Viral loads of various organs in the inoculated chickens throughout the trial.

Organs	Log_{10} Observed Reaction (Copies/titer)						
	Days post inoculation (dpi)						
	1	3	5	7	10	14	21
Bursa of Fabricius	11.286	12.414	11.668	11.037	10.955	9.702	9.623
Spleen	9.327	9.506	10.170	9.836	9.573	8.748	8.178
Thymus	8.214	10.109	9.800	9.213	9.583	8.613	7.694
Bone marrow	7.111	7.557	7.745	7.298	Not detected	Not detected	Not detected
Caecal tonsil	8.627	9.767	9.266	9.971	8.102	7.839	7.484