UNIVERSITI PUTRA MALAYSIA

EFFICACY AND SAFETY OF INACTIVATED NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN BROILER CHICKENS

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EFFICACY AND SAFETY OF INACTIVATED NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN BROILER CHICKENS

LATHASHA GAUTHAMAN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Efficacy and safety of inactivated novel variant infectious bursal disease virus in broiler chickens” by Lathasha Gauthaman and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD4999 – Final Year Project.

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoabsorbent assay
HI	Hemagglutination inhibition
HVR	Hypervariable region
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
MAB	Monoclonal antibodies
ND	Newcastle disease
nVarIBDV	Novel variant infectious bursal disease virus
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
vvIBDV	Very virulent infectious bursal disease virus

ABSTRAK

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

KEBERKESANAN DAN KESELAMATAN VIRUS PENYAKIT BURSAL BERJANGKIT VARIAN NOVEL YANG TIDAK DIAKTIFKAN DALAM AYAM PEDAGING

Oleh

Lathasha Gauthaman

2022

Penyelia: Professor Dato' Dr Mohd Hair bin Bejo

Penyelia bersama: Dr Mazlina binti Mazlan

Virus penyakit bursal berjangkit (IBDV) ialah jangkitan virus akut yang sangat menular yang menyebabkan kematian dan imunosupresi yang tinggi dalam ayam yang terdedah dan kekal sebagai ancaman utama kepada industri ayam global. Laporan terbaru tentang varian baru IBDV (nVarIBDV) di ladang ayam di China, Jepun, Korea Selatan dan Malaysia telah menunjukkan keperluan untuk membangunkan vaksin baharu terhadap strain ini kerana vaksin komersial yang ada tidak dapat melindungi ayam daripada jangkitan nVarIBDV dengan secukupnya. Kajian ini telah menilai keberkesanan dan keselamatan nVarIBDV yang tidak aktif sebagai calon vaksin yang berpotensi dalam ayam pedaging. Sebanyak 65 ekor ayam berumur sehari telah dibahagikan kepada tiga kumpulan iaitu A, B dan C. Mereka telah diimunisasi pada usia sehari (Kumpulan A dan B) dan penggalak pada hari ke-14 (Kumpulan B) dengan nVarIBDV yang tidak aktif (10^7 EID_{50}/0.2 ml). Kumpulan C bertindak sebagai kumpulan kawalan dan kekal tanpa inokulasi. Ayam dalam kumpulan yang dicabar (ACH, BCH dan CCH) telah dicabar dengan nVarIBDV 10^5 EID_{50}/1.0ml pada hari ke-28 selepas inokulasi. Berat badan dan bursa serta sampel darah ayam telah diambil. Lesi kasar direkodkan dan sampel bursa Fabricius diambil untuk penilaian histologi. Kajian menunjukkan

bahawa ayam itu sihat dan normal sepanjang kajian. Bagi semua kumpulan, berat badan meningkat sepanjang kajian sehingga hari ke-35, tanpa kepentingan statistik. Berat bursa dan nisbah bursa kepada berat badan kumpulan penggalak adalah ketara lebih tinggi ($p<0.05$) berbanding kumpulan bukan penggalak dan kawalan. Tiada perbezaan kasar dan ketara ($p>0.05$) markah lesi busa di kalangan semua kumpulan. Titer antibodi kumpulan ACH, BCH dan CCH adalah lebih tinggi daripada ($p>0.05$) kumpulan A, B, dan C tetapi tidak signifikan. Titer antibodi kumpulan BCH adalah jauh lebih tinggi ($p<0.05$) daripada kumpulan ACH dan BCH. Oleh itu, disimpulkan bahawa nVarIBDV yang tidak aktif adalah selamat dengan dos penggalak (Kumpulan B) mempunyai keberkesanan yang lebih tinggi dan mampu mendorong tindak balas imun humoral.

ABSTRACT

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

EFFICACY AND SAFETY OF INACTIVATED NOVEL VARIANT INFECTIOUS BURSAL DISEASE VIRUS IN BROILER CHICKENS

By

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2022

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Infectious bursal disease virus (IBDV) is an acute, highly contagious viral infection that causes high mortality and immunosuppression in susceptible chickens and remains as a major threat to the global poultry industry. Recent reports of a novel variant IBDV (nVarIBDV) in poultry farms in China, Japan, South Korea and Malaysia have indicated the need to develop a new vaccine against this strain as the available commercial vaccines are unable to sufficiently protect chickens from the nVarIBDV infection. This study focused on evaluating the efficacy and safety of inactivated nVarIBDV as a potential vaccine candidate in broiler chickens. A total of 65 day-old chickens were divided into three groups namely A, B, and C. They were immunized at day-old (Groups A and B) and booster at day 14 (Group B) with inactivated nVarIBDV (10^7 EID_{50}/0.2 ml). Group C acted as the control group and remained uninoculated. The chickens in the challenged groups (ACH, BCH and CCH) were challenged with nVarIBDV 10^5 EID_{50}/1.0ml on day 28 post inoculation. The body and bursa weights and blood samples of the chickens were collected. Gross lesions were recorded and samples of bursa of Fabricius were collected for histological evaluation. The study showed that the chickens were healthy and normal throughout the trial. For all groups, the body weight increased throughout the trial

until day 35, without statistical significance. The bursa weight and the bursa to body weight ratio of the booster group was significant higher ($p<0.05$) than the non-booster and control groups. No gross and significant difference ($p>0.05$) of busa lesions scoring among all the groups. The antibody titre of groups ACH, BCH and CCH were higher than ($p>0.05$) groups A, B, and C but not significant. The antibody titre of group BCH was significantly higher ($p<0.05$) than groups ACH and BCH. Thus, it was concluded that the inactivated nVarIBDV is safe with booster dose (Group B) has a greater efficacy and able to induce humoral immune response.

1. INTRODUCTION

1.1 Background

Infectious bursal disease (IBD) is an acute, highly contagious viral infection caused by the infectious bursal disease virus (IBDV), a member of the genus *Avibirnavirus* of the *Birnaviridae* family. This disease is also known as the “Gumboro disease” owing to the occurrence of first outbreaks in the Gumboro region of Delaware, United States (Cosgrove, 1962). IBD primarily affects young chickens with greater detrimental effects towards the lymphoid tissue with a special predilection for the bursa of Fabricius. Clinical manifestation of this disease varies according to the virus strain causing infection and both have a significant impact on the poultry industry and economy. Some virus strains are highly virulent and may cause up to 20% or more mortality in chickens 3 weeks of age and older whereas a second group of strain can cause a severe, prolonged immunosuppressive reaction in chickens infected at an early age (Saif and Fadly, 2008).

IBDV is a single-shelled, non-enveloped virion with icosahedral symmetry and a diameter varying from 55-65 nm. The dsRNA of IBDV genome has two segments known as genome A and genome B which encode five different viral proteins designated as VP1, VP2, VP3, VP4, and VP5 (Dobos et al., 1979; Muller et al., 1979). VP2 and VP3 are the major structural proteins of IBDV as they were identified in western-blotting experiments with convalescent sera as important IBDV-derived antigens. IBDV has been classified into 2 serotypes, serotype I and serotype II. Serotype I viruses are pathogenic to chickens while serotype II viruses are non-pathogenic to chickens, and can be found in chicken and turkey sera (Jackwood et al., 1994). Therefore, immunization against serotype II viruses does not protect against serotype I. Serotype I viruses are further classified into four different subtypes which are the classical IBDV (caIBDV), variant IBDV (varIBDV), very virulent IBDV (vvIBDV) and attenuated IBDV (attIBDV) strains (Muller et al., 2003). Besides that, new and improved ways for the classification based on VP1 and VP2 characteristics have been proposed (Wang et al., 2021).

Based on the improved scheme, caIBDV, varIBDV, vvIBDV and attIBDV are classified as Genotype A1B1, A2B2 (including A2aB1, A2bB1, and A2cB1), A3B2 and A8B1, respectively.

Since 2017, a novel variant IBDV (nVarIBDV), which is genetically different from the early varIBDV has become widely prevalent in immunized chicken farms in China (Wang et al., 2021). It was first reported in Malaysia in 2019 (Aliyu et al., 2021). Although this variant does not immediately result in mortality, it has a high morbidity and causes severe atrophy of the bursa of Fabricius. This leads to immunosuppression, loss in production performance and subsequently, severe economic losses (Fan et al., 2019b, 2020; Xu et al., 2019). The nVarIBDV is classified under Genotype A2dB1 and was also successively reported in Japan (Myint et. Al, 2021) and South Korea (Thai et al., 2021). Recently, it was also described that the Chinese nVarIBDV and the early variant IBDV originally found in America belong on the same branch of variant IDBV although they still divided to form two distinct sub-branches discrete to one another (Aliyu et al., 2021).

Due to the threat of economic impact of nVarIBDV on infected chickens, protection of chicks is crucial to prevent immunosuppression and production loss. However, nearly all commercial vaccines currently in use target vvIBDV, and do not mount a sufficient immune response against nVarIBDV (Wang et al., 2021). Thus, it is essential to develop antigenicity matching vaccines to a complete and effective prevention and control of nVarIBDV in the poultry industry. In another recent publication, a viral-particle like vaccine candidate, SHG19-VLP vaccine was able to elicit neutralization antibodies and provide 100% protection against the nVarIBDV (Wang et al., 2021). Besides that, an attenuated vaccine strain Gt was used to develop a reassortment virus strain rGtVarVP2 was shown to provide complete protection on the nVarIBDV (Fan et al., 2020). In this study, inactivated nVarIBDV is to be evaluated for its potential to be a vaccine candidate by assessing its safety and efficacy in broiler chickens.

1.2 Justification

The justifications of the study were:

1. To develop a new vaccine against nVarIBDV due to the failure of current commercial vaccines to target this strain.
2. To prevent immunosuppression in chickens infected with nVarIBDV.
3. To prevent interference of nVarIBDV with the vaccination of other diseases.
4. To prevent economic losses caused by nVarIBDV.

1.3 Hypothesis

The hypotheses of the study were:

Hypothesis 1

H_o: There is insufficient safety and efficacy of inactivated nVarIBDV in broiler chickens

H_a: There is sufficient safety and efficacy of inactivated nVarIBDV in broiler chickens.

Hypothesis 2

H_o: There is no difference in the efficacy and safety of inactivated nVarIBDV in broiler chickens immunized with a booster dose compared with a single dose.

H_a: There is greater efficacy and safety of inactivated nVarIBDV in broiler chickens immunized with a booster dose compared with a single dose.

1.4 Objectives

Main objective

To evaluate the efficacy and safety of inactivated nVarIBDV in broiler chickens.

Specific objective

To determine the clinical signs, gross and histological lesions, IBD antibody titer, viral load and viral shedding of broiler chickens inoculated with inactivated nVarIBDV and challenged with pathogenic field isolate of nVarIBDV.

2.0 LITERATURE REVIEW

2.1 Background

In 1957, IBD was first reported in Gumboro, Delaware, USA after which it is also commonly referred to as the Gumboro disease (Cosgrove, 1962). Rosenberger and Cloud reported in 1985 that a new outbreak of IBD emerged in 1984 despite a well-controlled vaccination programme, and these new isolates were called variants. Around this time, the first reports of very virulent IBDV strains were made in Netherlands (Chettle et al., 1989).

2.2 Emergence of nVarIBDV in China and around the world

A study of 365 clinical samples of the bursa of Fabricius from broilers in 76 flocks from 6 provinces in China showed that the samples that were positive for a nVarIBDV that belonged in the phylogenetic tree VP2 and had representatives from one branch referred to as nVarIBDVs (Fan et al., 2019). The Chinese variants amino acid sequence showed a similarity to Variant E, E/Del, Variant A and 9109, and GLS.

In Japan, Myint et al., (2021), conducted an analysis of mortality to bursa and body weight ratio where the phylogenetic analysis indicated new antigenic variant IBDV strains that are highly homologous to the China isolates. The study suggested the possibility for the origin of these strains may be a transmission from China.

In South Korea, a study on 29 chicken carcasses under passive surveillance for IBDV showed that 5 of them carried nVarIBDV. Their phylogenetic analysis of hypervariable region (HVR) of VP2 nucleotide sequence revealed several isolates. These isolates were clustered into new variant sub-lineage which emerged recently in China (Thai et al., 2021).

In Malaysia, a study of a total of 30 flocks of chickens suspected having IBDV infection in five states of Malaysia between 2017 and 2019 revealed that amino acids sequencing and phylogenetic analyses for HVR for all the 11 positively tested isolates (UPM1432/2019 and UPM1219/2019) were identical to genogroup 2 (G2). Based on the phylogenetic tree of the

HVR of isolates UPM1432/2019 and UPM1219/2019, they formed a distinct cluster with nVarIBDV (Aliyu et al., 2021). Based on the phylogeny formed by the study, UPM1432/2019 is considered a variant strain as it was clustered with Chinese and American variants of IBDV (Aliyu et al., 2021).

2.3 Clinical signs and gross lesions

The chickens infected with nVarIBDV showed absence of clinical signs and gross lesions except the body weight was negatively influenced (Fan et al., 2021). Overall, nVarIBDV caused no obvious clinical signs and mortality. Myint et al., (2021) reported sneezing observed in chickens at 21 dpi, but it was later ruled out as a secondary bacterial infection. Furthermore, there are no concrete evidence that nVarIBDV infection can cause slight sneezing as most of the studies shows only immunosuppressive and bursa atrophy as the lesions. Upon necropsy, bursa atrophy, bursal haemorrhage with yellowish exudation with initial splenomegaly followed by splenic atrophy were observed at 5 and 25 dpi, respectively (Fan et al., 2021). In comparison, Chinese nVarIBDV causes a greater extent of lesions compared to the subclinical American varIBDV as the China isolate nVarIBDV have an additional lesion of yellowish exudation in bursa of Fabricius and splenic atrophy.

2.4 Histopathological changes

Histopathological changes of the bursa of Fabricius induced by nVarIBDV shows similar lesions among the American and Chinese variants. The lesions include destruction of lymphocytes, minimal to no inflammatory response, severe follicular lymphoid necrosis and depletion, and multifocal follicular lymphoid infiltration. Few other lesions recorded include reticular and macrophage infiltration in lymphoid follicles, cystic cavities in lymphoid follicles, proliferation of fibrous tissues, severe follicular atrophy, and infolding epithelium into damaged follicles (Fan et al, 2021; Myint et al., 2021; Thai et al, 2021). This shows that nVarIBDV is pathogenic to susceptible chickens and show similar lesions as vvIBDV. Chickens infected with vvIBDV also showed severe haemorrhages, lymphoid necrosis, lymphocyte depletion,

development of cystic cavities in bursal follicles, erosion of bursal epithelium and presence of fibrous tissue between bursal follicles during histopathological examination (Pang et al., 2015; Singh et al., 2015).

2.5 Antigenic distinction between nVarIBDV and vvIBDV

Fan et al., (2020) performed the MAB reactivity pattern of IBDV to detect the antigenic differences between nVarIBDV and vvIBDV. The results indicated that nVarIBDV showed different MAB reactivity pattern from vvIBDV. Furthermore, the antigenic mismatch of nVarIBDV and vvIBDV were confirmed using cross-neutralization assays. The study indicated obvious antigenic difference between two virus strains. The nVarIBDV strain showed antigenic differences from vvIBDV strains with R values of 0.64 and 0.35, respectively (Fan et al., 2020). This showed that antigenic variation and mismatch are primary factors involved in immune failure which leads to emergence of nVarIBDV in a large-scale.

2.6 Immune failure of Newcastle Disease vaccination induced by nVarIBDV

Fan et al., (2020) conducted a study to evaluate the effects of nVarIBDV on Newcastle Disease vaccination in broiler and layer chickens. The results showed that the nVarIBDV strain had severely destroyed the bursa, which is important for the induction of humoral immune responses in chickens. Therefore, chickens infected with nVarIBDV decreased HI antibody titres against ND vaccine due to the destruction of B lymphocytes in the bursa (Fan et al., 2020). It was also reported that an early infection of one-day-old chickens with variant IBDV might induce more severe suppression of antibodies against avian influenza virus (AIV) vaccine (Spackman et al., 2018).

2.7 Trial vaccines against the nVarIBDV

A study used Lactococcus lactis (L. lactis), a food-grade probiotic with non-pathogenic, non-invasive, and non-colonizing properties as a vector to express the nVarIBDV VP2 protein. Using the nisin-controlled gene expression system, recombinant L. Lactis (inactivated r-L. lactis-avVP2-RCK) was used for injection immunization of SPF chickens against nVarIBDV.

The results showed that the immunization induces the production of unique specific neutralizing antibodies, which provide complete protection of nVarIBDV (Wang et al., 2020). These results also suggest that r-L. lactis-avVP2-RCK induces a unique VP2 neutralizing antibody that is different from those produced by other vaccines, thus cannot be detected by conventional IBD antibody ELISA kits. Furthermore, chickens challenged with vvIBDV exhibit a 100% survival rate (Wang et al., 2020).

A viral-like particle of nVarIBDV was developed using prokaryotic expression system which was evaluated as a vaccine candidate in vitro and in vivo. In this study, the recombinant protein nVarIBDV was expressed successfully using *E. coli* to create a viral-like particle vaccine. The results of this research showed that the candidate vaccine can provide complete immune protection not only against the homologous nVarIBDV but also the heterologous vvIBDV (Wang et al., 2021).

3.0 MATERIALS AND METHODS

3.1 Experimental Design

Sixty-five day-old commercial broiler chicks were randomly divided into six groups; A, ACH, B, BCH, Cand CCH (Appendix 1). At day-old, chickens from Groups A, ACH, B and BCH were inoculated (0.2ml) with 10^7 EID_{50}/0.2 ml of inactivated nVarIBDV via subcutaneous route. Chicks from Groups C and CCH were not inoculated. Five chicks from the Group C were sacrificed for data collection. At 14-day-old, 0.2ml of 10^7 EID_{50}/0.2 ml inactivated nVarIBDV were inoculated to chickens from Group B and BCH via the subcutaneous route. Five chickens from Groups A, B and C were sacrificed at days 14, 28 and 35 for data collection. All the chickens were observed daily for any clinical sign abnormalities throughout 35 days of the trial. At 28-day old, chickens from Groups ACH, BCH and CCH were challenged with 10^5 EID_{50}/1.0ml of pathogenic field strain of nVarIBDV via ocular (0.2ml) and oral (0.8ml) routes. The chickens were monitored throughout 7 days post challenged. Necropsy were conducted in any dead chickens and the chickens that survived after the challenged will be sacrificed for data collection. Body weight and blood samples for detection of IBD antibody using ELISA technique were collected prior to sacrifice. On necropsy, the gross lesions and bursa of Fabricius weight were recorded. Samples of the bursa and cloacal swabs were collected for detection of the challenged virus using RT- qPCR. Bursa samples were fixed in 10% buffer formalin for histological examination and lesion scoring.

3.2 Candidate Vaccine Preparation and Vaccination Method

Six ml of nVarIBDV isolate was measured into a centrifuge tube and 120 µl of binary ethylene inime (BEI) was added as an inactivating agent. The mixture containing the isolate and inactivating agent was incubated at 37^{o}C for 36 hours and vortexed every 30-minute interval for thorough mixing. After 36 hours, 12 µl of sodium thiosulfate was added to halt the action of the inactivating agent and mixed thoroughly with vortex mixer at 37^{o}C for one hour. The inactivated nVarIBDV isolate was then filtered through a 0.22 µm syringe filter to remove any contaminant and mixed with Montanide 71 VG which acts as an adjuvant at a ratio of 30:70

(inactivated nVArIBDV: Montanide 71 VG). The inactivated nVarIBDV isolate with Montanide 71 VG was mixed by votexing for 2 hours and stored at 4°C for use as inoculum. To ensure the prepared inactivated nVarIBDV isolate meets safety and sterility requirements, a test was conducted by inoculating 0.1ml of the isolate into specific-pathogen free eggs through the chorioallantoic membrane route and incubated at 37°C for 7 days. The method of vaccination was via the subcutaneous route and done at the loose skin of the dorsal neck of the chicks.

3.3 Antibody Titer

Serum samples collected from chickens at different ages were tested for nVarIBDV antibodies using commercial enzyme-linked immunoabsorbent assay (ELISA) kit (BioCheck IBD ELISA, Hounslow, UK). Antigen-coated plate was acclimatized to room temperature prior usage. One hundred µL of negative and positive controls were dispended into the respective wells accordingly. After that, 100 µL of 1:500 (v/v) diluted test sera were dispensed into the respective wells. Plate was covered and incubated at room temperature for 30 minutes. The plate was then washed 4 times with 300µL wash buffer per well. After washing, 100µL of sheep anti-chicken IgG labeled with alkaline phosphatase was added into the well, and further incubated at room temperature for 30 minutes. The plate was washed as described previously and 100µL of substrate buffer that contained diethanolamine buffer with enzyme co-factor was added into the well. Plate was incubated for another 15 minutes at room temperature. One hundred µL of stop solution was dispensed into each well to stop the reaction. Microtitre plate reader was used to record the absorbance at 405nm and the nVarIBD antibody titer was then generated using BioCheck 2000 software.

3.4 Polymerase Chain Reaction

Bursa and cloacal swab samples were pooled in sterile buffer and soaked for 24 hours. Then, the sample were washed out thoroughly by pulse-vortexing and the supernatant was used. Twenty mg tissue per purification was used. Then, 200 µl lysis solution was added to a Kylt® Lysis tube. After that, 200 µl of sample was added. Ten µL of liquid proteinase K was added to a lysis tube. The mixture was pulse-vortexed and spinned down briefly. The mixture was

then incubated at room temperature for 5 minutes and at 70°C for another 5 minutes. The sample was allowed to cool down for 2 minutes. For binding, 200 µL of ethanol 96% was added. The mixture was pulse-vortexed and spinned down briefly. The mixture was transferred to Kylt® Binding column and centrifuged for 1 minute at 10,400 rpm. The collection tube was discarded and a new collection tube was attached. Then, 500 µL of wash solution was added to the Kylt® Binding column and centrifuged for 1 minute at 10,400 rpm. The collection tube was discarded and a new collection tube was attached. Then 500 µl of wash solution 2 was added to the Kylt® Binding column and centrifuged at 2 minutes for 14,000 rpm (maximum speed). The collection tube was then discarded and the elution tube was placed on the column. Then 100 µL of elution buffer was added directly to the membrane without touching it. The mixture was incubated for 1 minute at 70°C and centrifuged for 1 minute at maximum speed. The Kylt® Binding column was discarded and the elution tube was closed. The eluate contains the purified RNA and DNA. After extraction of nucleic acid, RT-qPCR assay was run where specific primers were used to amplify the purified nucleic acid and quantify the viral DNA copies in the samples.

3.5 Histopathology and Lesion Scoring

Bursa of Fabricius was removed from the body during post mortem and fixed in 10% buffered formalin solution for 24 hours. Tissue samples were then trimmed to a thickness of 5mm and subjected to a series of dehydration, clearing and impregnation processes using an automatic machine (Leica ASP 300). Processed samples were embedded within paraffin wax, sectioned to 4µm and fixed on glass slides for haematoxylin and eosin staining. Bursa samples were examined for histological changes under a light microscope. Histological changes were graded on a scale of 0 to 5 where; 0 (normal), 1 (mild), 2 (mild to moderate), 3 (moderate), 4 (moderate to severe) and 5 (severe) (Appendix 2).

3.6 Statistical Analysis

Data collected were analyzed using SPSS version 28.0.

4.0 RESULTS

4.1 Clinical signs

Throughout the 35 days of trial, all the chickens were normal and healthy. There were no abnormal clinical signs and no mortality observed (Figure 1).

Figure 1: Normal clinical signs of chickens. (a) Group C, day 0 pi. (b) Group A, day 14 pi. (c) Group A, day 28 pi. (d) Group B, day 35 pi. (e) Group B, day 35 pi (CH).

4.2 Body weight

The body weight of chickens increased until day 35 without statistical significance for all the Groups A, B and C. For day 14, there was no statistically significant difference ($p>0.05$) of body weight between the groups. For day 28, there was a statistically significant difference ($p<0.05$) of body weight between the groups. For day 35, there was no statistically significant difference of body weight between the three groups for both challenged and non-challenged chickens. The body weight of challenged chickens in Group B was significantly higher ($p<0.05$) than the non-challenged. Among the challenged chickens, Group B had the highest body weight than Groups A and C, but not statistically significant ($p>0.05$) (Figure 2; Appendix 3).

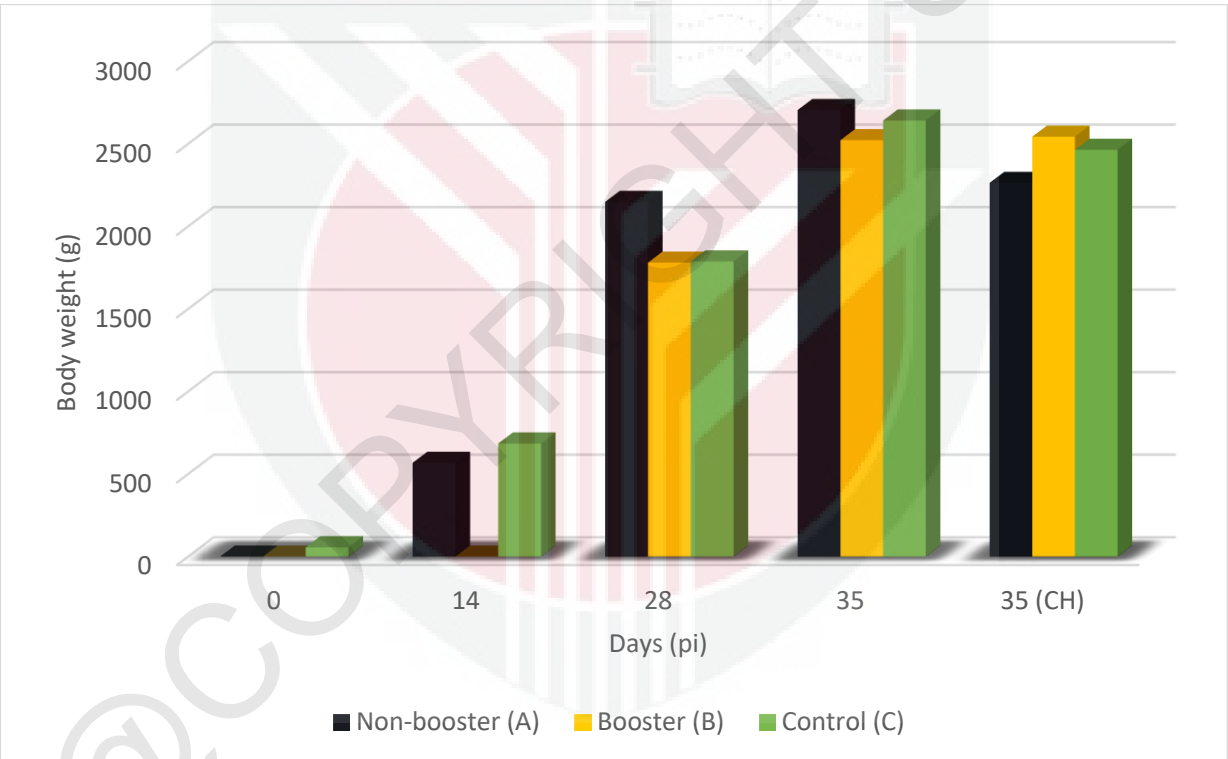

Figure 2: Body weight of chickens throughout the trial.

4.3 Bursa weight

There was no statistically significant difference in the bursa weight between the groups on days 14, 28 and 35 of the non-challenged chickens. For day 35 of the challenged group, bursa weight of challenged chickens in Group B was significantly higher ($p<0.05$) than Groups A and C (Figure 3; Appendix 4).

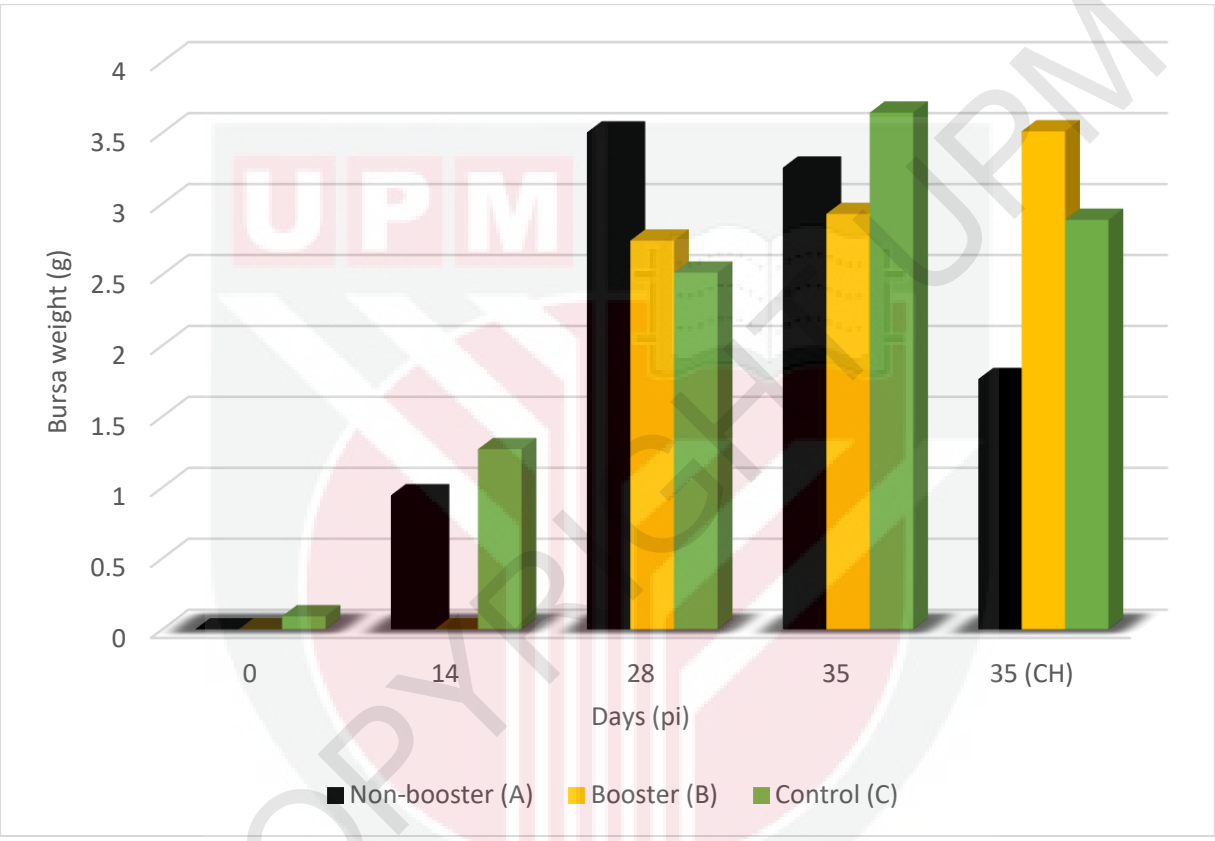

Figure 3: Bursa weight of chickens throughout the trial.

4.4 Bursa to Body weight ratio

For all groups, the bursa to body weight ratio was not significantly different ($p>0.05$) except on day 35 among challenged chickens. The bursa to body weight ratio of challenged chickens in Group B was significantly higher ($p<0.05$) than Groups A and C (Figure 4; Appendix 5).

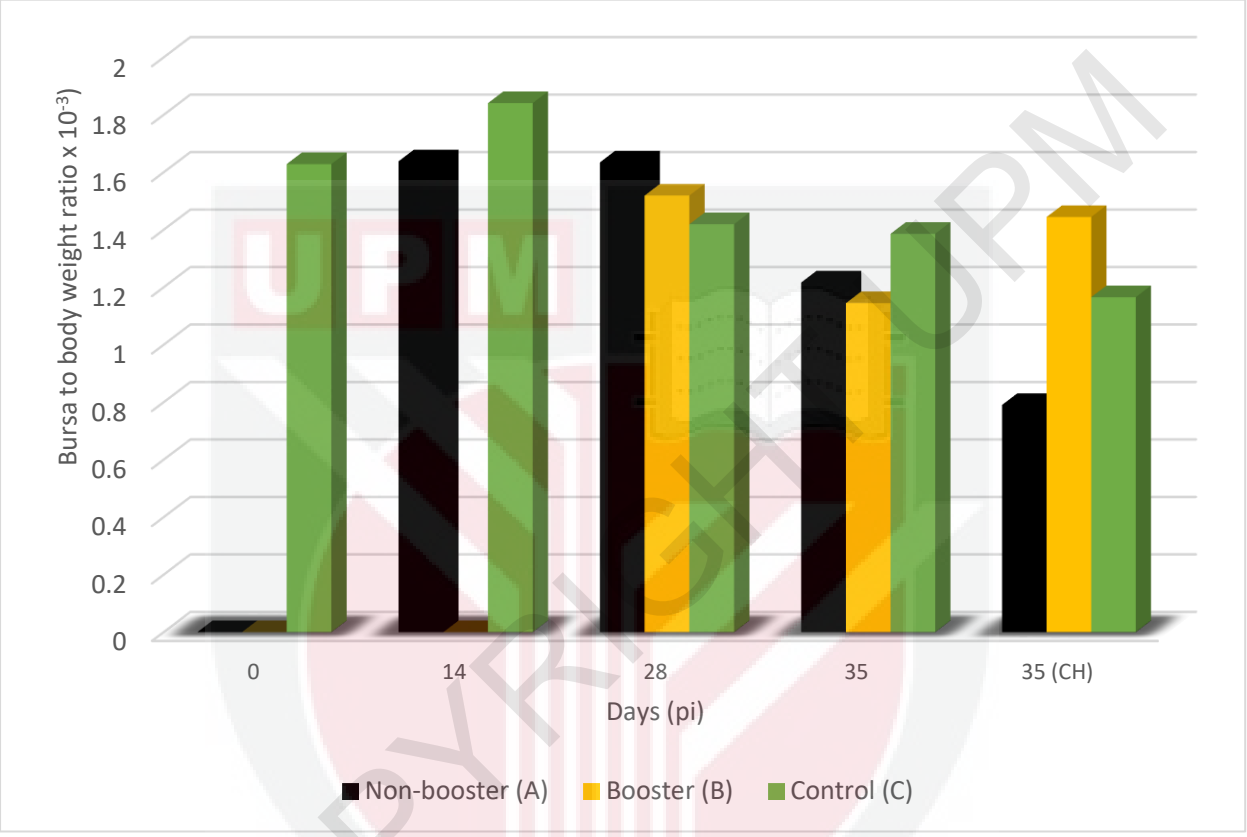

Figure 4: Bursa to body weight ratio of chickens throughout the trial.

4.5 Gross lesions

4.5.1 Day 0 pi

The bursa of day-old chicks (DOC) was normal with no gross lesions (Figure 5).

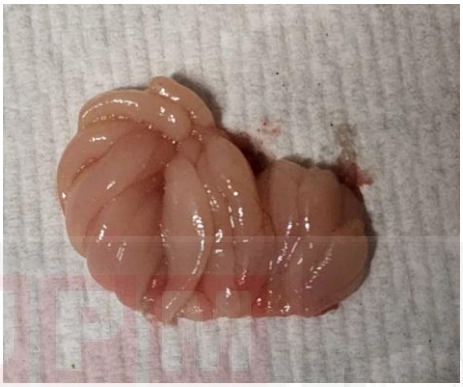

Figure 5: Normal bursa of Fabricius on day 0 pi (Group C).

4.5.2 Day 14 pi

The bursa from Groups A and B were normal with no gross lesions (Figure 6).

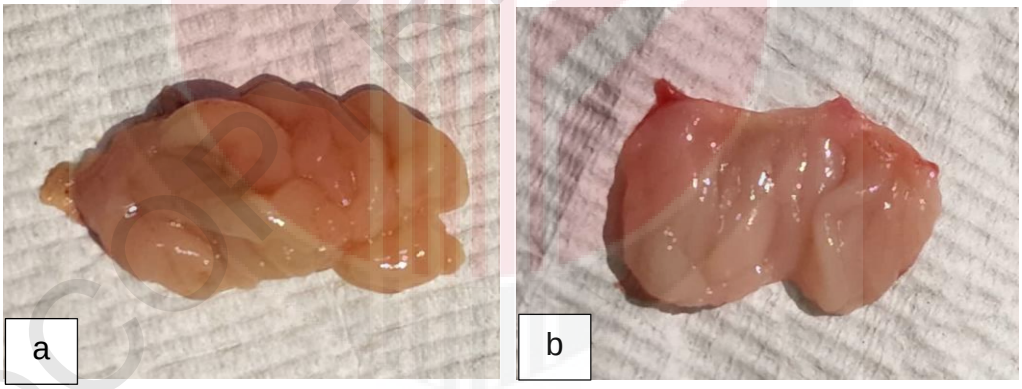

Figure 6: Normal bursa of Fabricius on day 14 pi. (a) Group A and (b) Group C.

4.5.3 Day 28 pi

The bursa from Groups A, B and C were normal with no gross lesions (Figure 7).

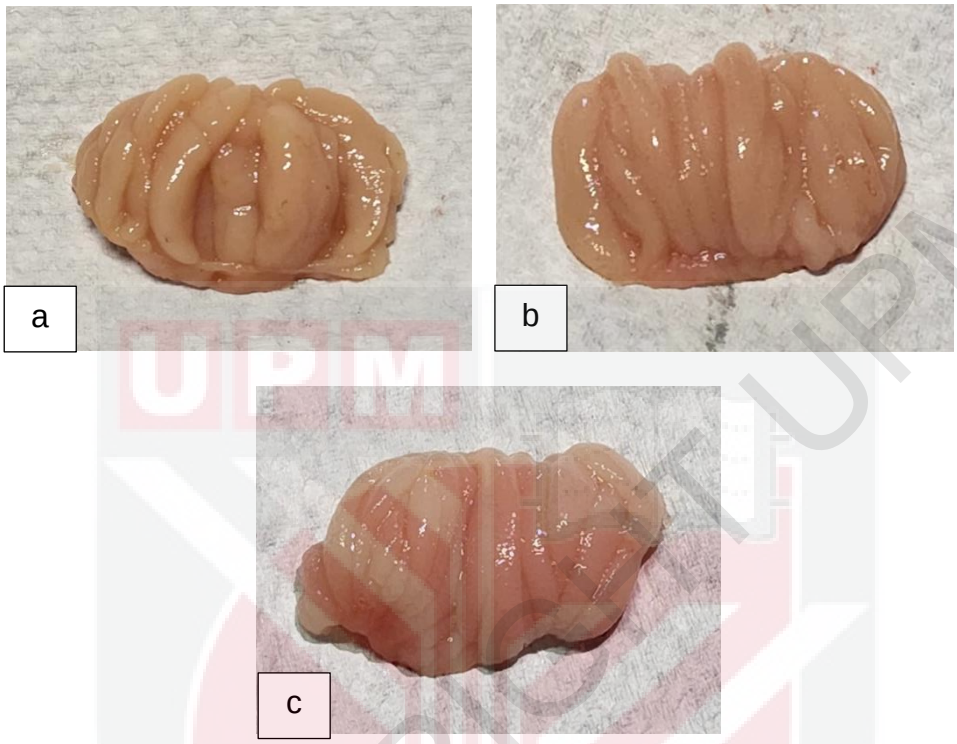

Figure 7: Normal bursa of Fabricius on day 28 pi. (a) Group A, (b) Group B and (c) Group C.

4.5.4 Day 35 pi

The bursa from Groups A, B and C were normal with no gross lesions (Figure 8).

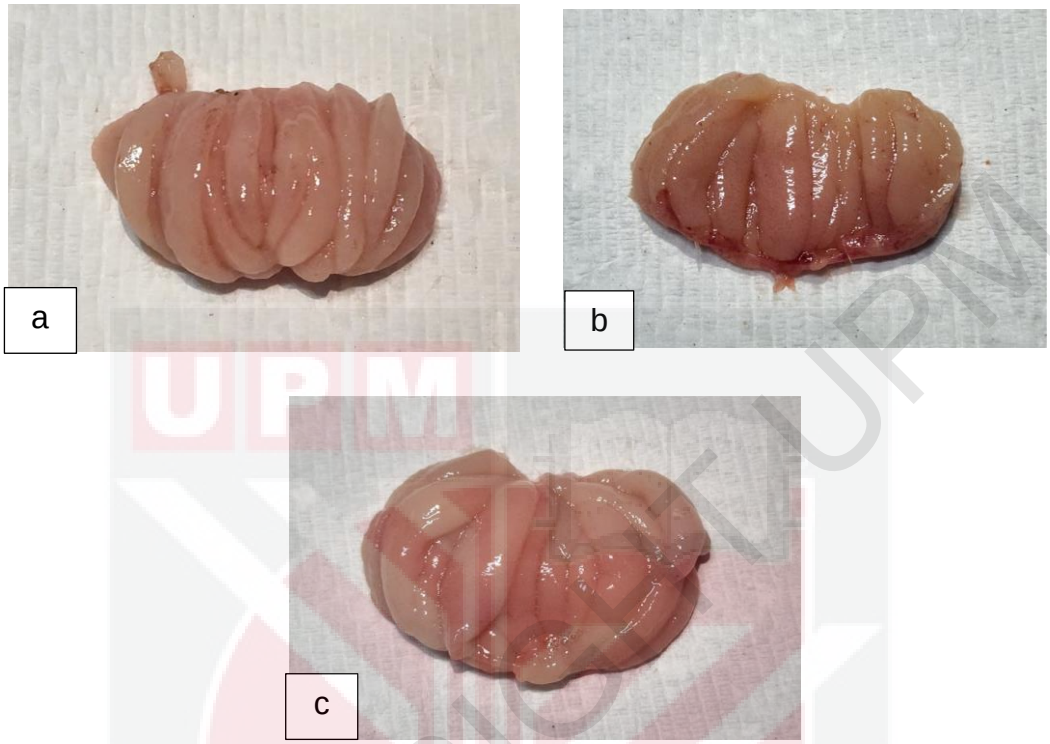

Figure 8: Normal bursa of Fabricius on day 35 pi. (a) Group A, (b) Group B and (c) Group C.

4.5.5 Day 35 (CH) pi

The bursa from Groups A, B and C were normal with no gross lesions (Figure 9).

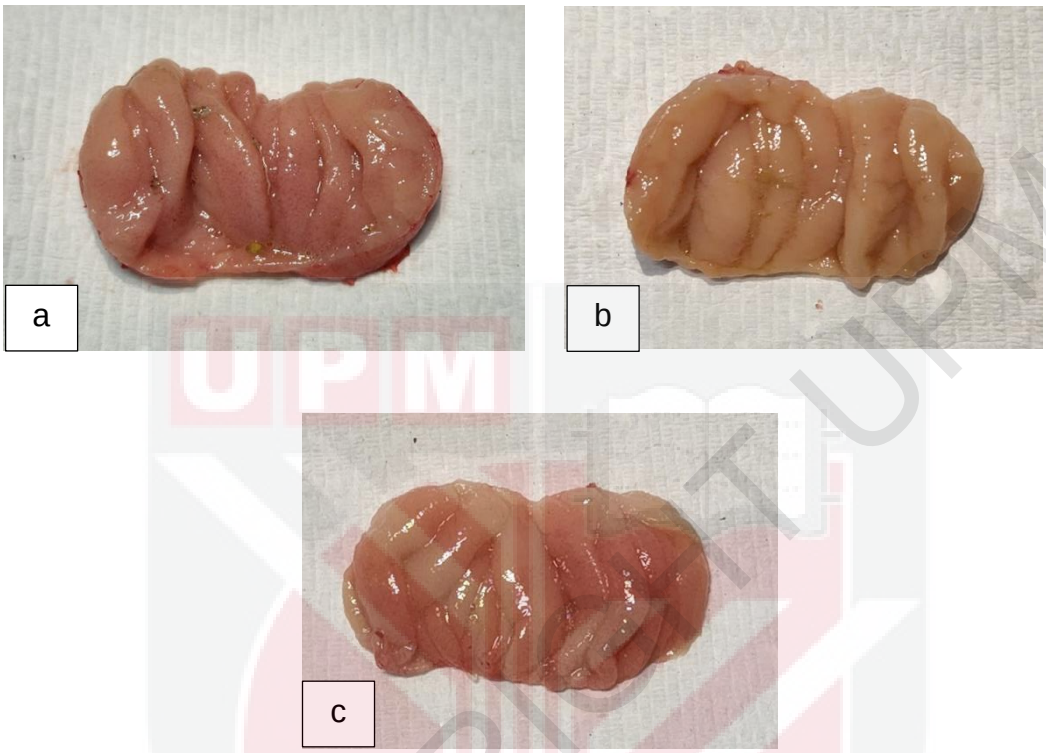

Figure 9: Normal bursa of Fabricius on day 35 pi (CH). (a) Group A, (b) Group B and (c) Group C.

4.6 Histological lesions

4.6.1 Day 0 pi

Presence of mild degeneration especially at the medullary region of the lymphoid follicles. No necrosis and heterophils were present (Figure 10).

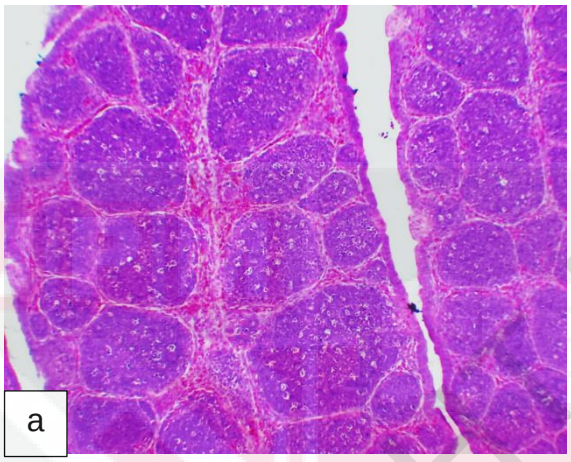

Figure 10: Histology of bursa of Fabricius on day 0 pi (Group C). Lesion scoring of 1. HE, 100x.

4.6.2 Day 14 pi

No histological lesions were observed from Groups A, B and C (Figure 11).

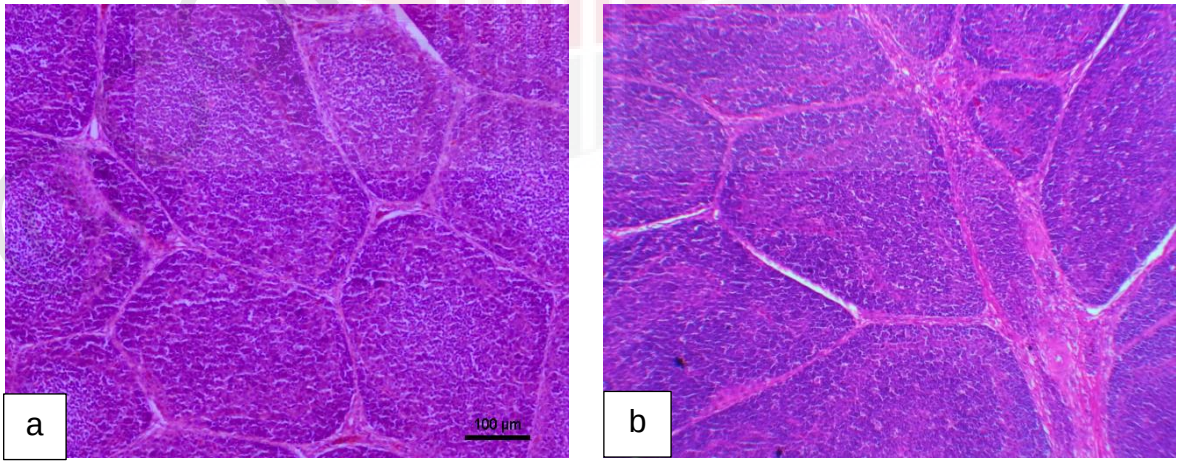

Figure 11: Normal histology of Bursa of Fabricius on day 14 pi. (a) Group A (Lesion scoring of 0), and (b) Group C (Lesion scoring of 0). HE, 100x, Bar=100µm.

4.6.3 Day 28 pi

No histological lesions were observed for Groups B and C. For Group A, mild degeneration especially at the medullary region of the lymphoid follicles were observed (Figure 12).

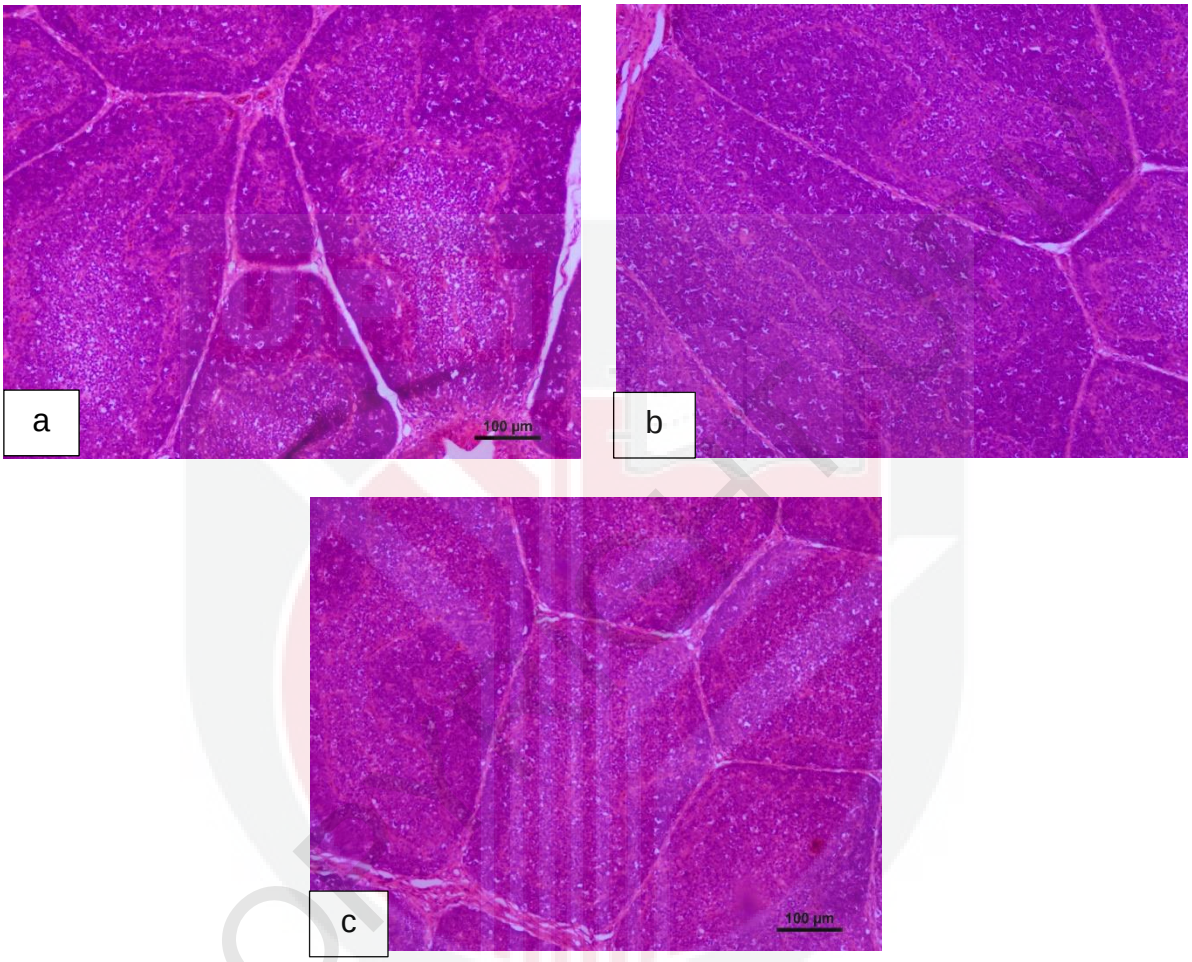

Figure 12: Histology of bursa of Fabricius on day 28 pi. (a) Group A (Lesion scoring 1), (b) Group B (Lesion scoring of 0) and (c) Group C (Lesion scoring of 0). HE, 100x, Bar=100µm.

4.6.4 Day 35 pi

No histological lesions were observed for Groups B and C. For Group A, mild degeneration especially at the medullary region of the lymphoid follicles were observed (Figure 13).

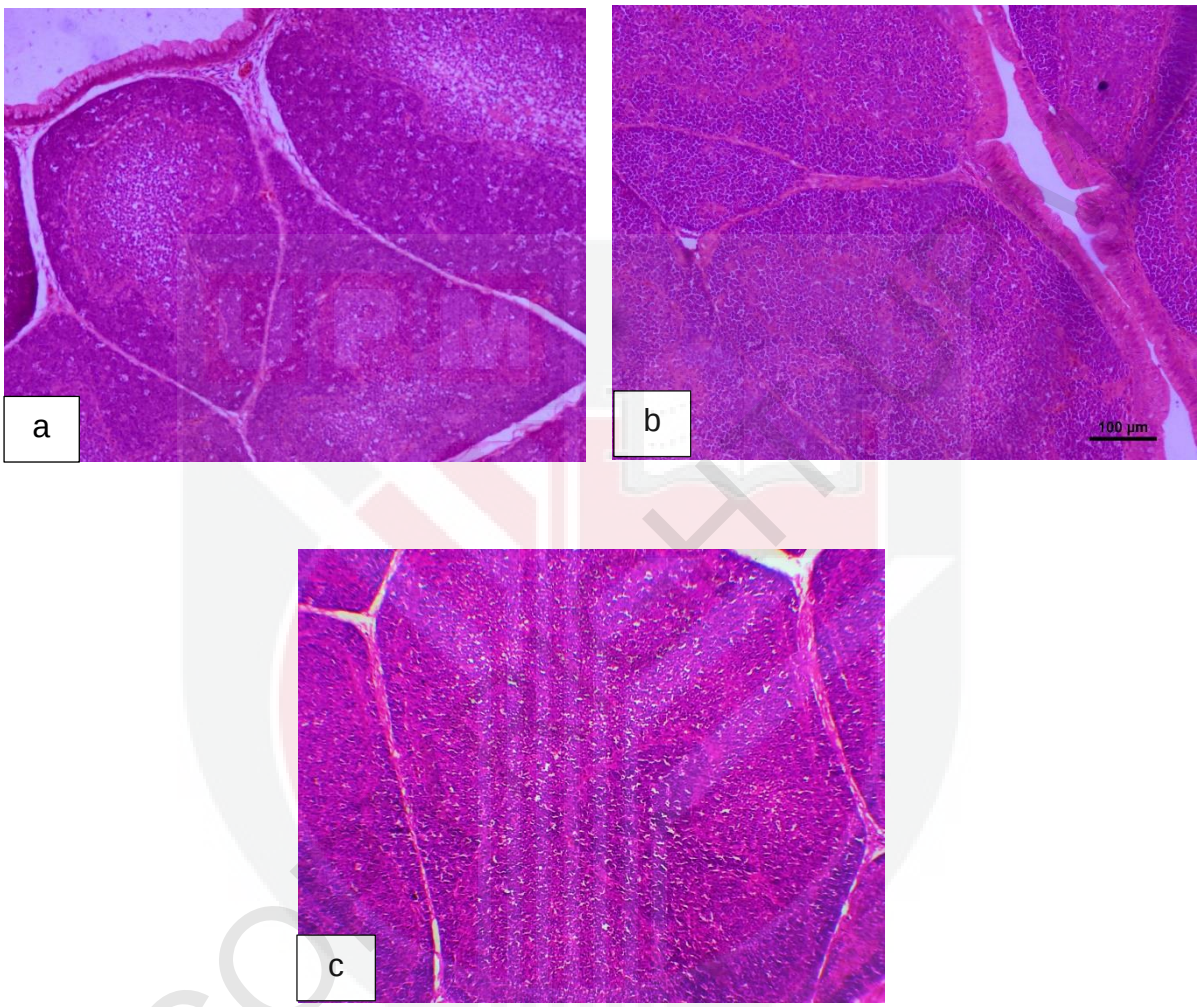

Figure 13: Histology of bursa of Fabricius on day 35 pi. (a) Group A (Lesion scoring of 1), (b) Group B (Lesion scoring of 0) and (c) Group C (Lesion scoring of 1). HE, 100x, Bar=100µm.

4.6.5 Day 35 pi (CH)

For Groups A and B, mild degeneration especially at the medullary region of the lymphoid follicles were observed. Bursa of Group C had mild to moderate degeneration and necrosis in the medulla and infiltration of inflammatory cells can be observed (Figure 14).

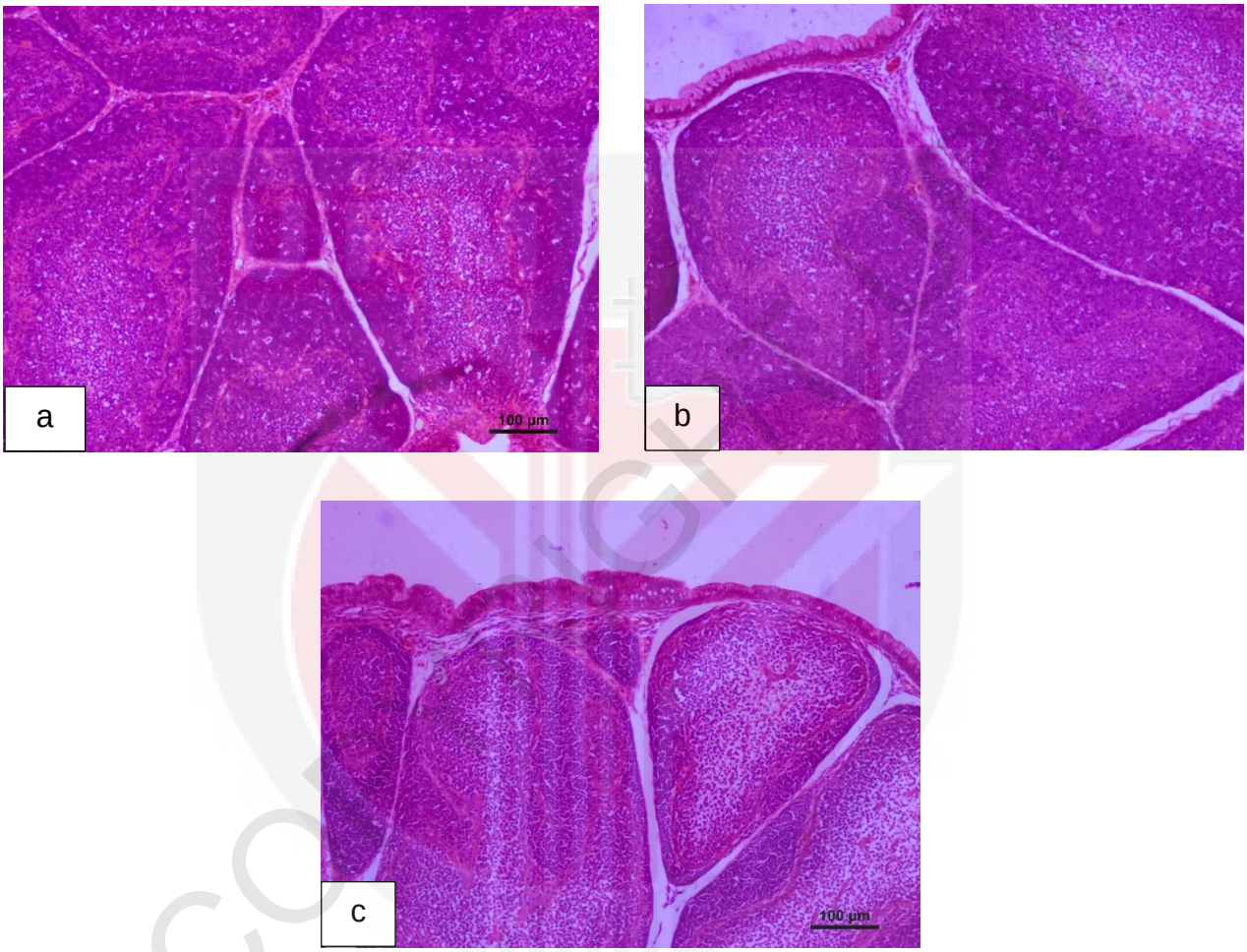

Figure 14: Histology of bursa of Fabricius on day 35 pi of the challenge groups (CH). (a) Group A (Lesion scoring 1), (b) Group B (Lesion scoring of 1) and (c) Group C (Lesion scoring of 2). HE, 100x, Bar=100µm.

4.7 Bursa lesion score

The bursa lesion score was not significantly different throughout the trial for all the groups. The bursa lesion score of challenged Group C was higher than Groups A and B but not statistically significant ($p>0.05$) (Figure 15; Appendix 6).

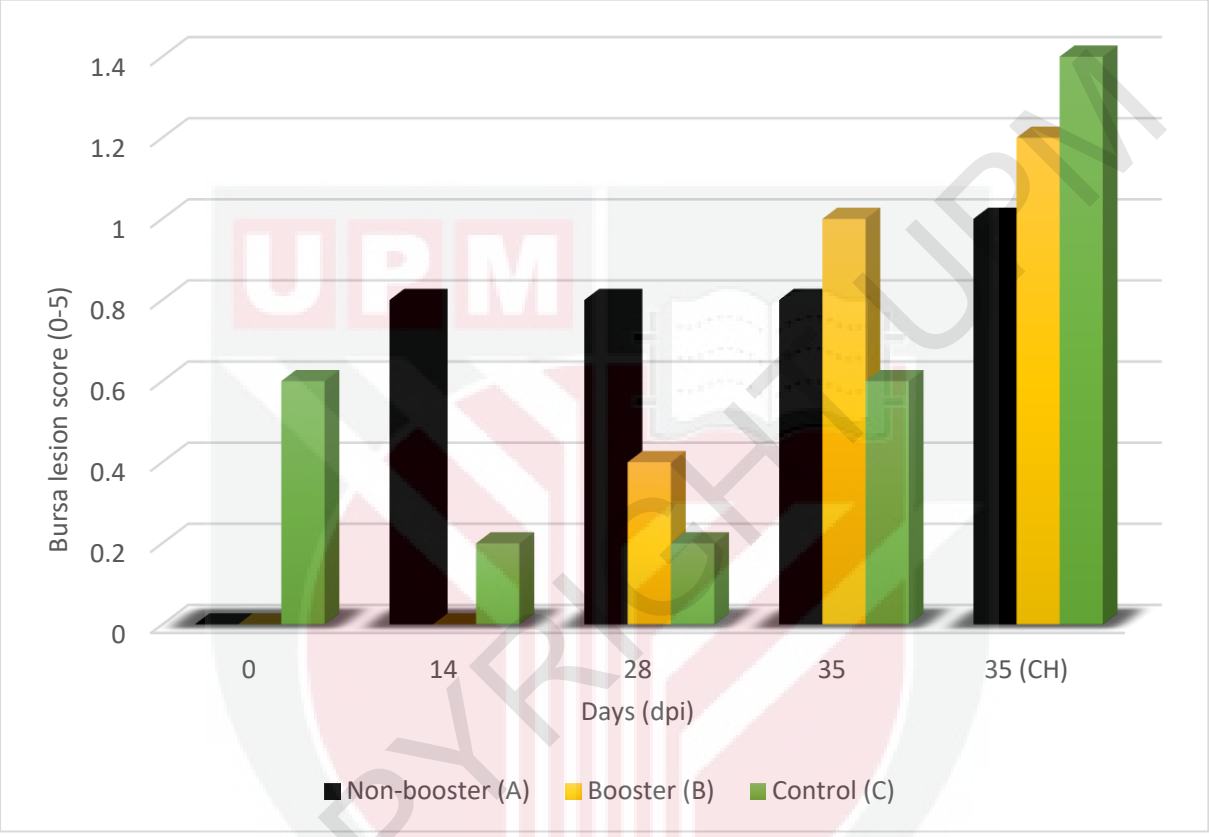

Figure 15: Bursa lesion score of chickens throughout the trial.

4.8 Virus loading and shedding (*RT-qPCR)*

The virus copies in the bursa and cloacal swab samples were higher in Group C when compared to the Groups A and B on day 35 pi or day 7 post challenged (Figure 16; Appendix 7).

Figure 16: Virus loading and shedding of chickens in the challenge groups (CH) on day 35 pi or day 7 post challenged.

4.9 IBD antibody titre

The IBD antibody titre of day-old chicks was 3546±556 ELISA unit. There was no significant difference in the antibody titre of the challenged and non-challenged groups ($p>0.05$). The antibody titer of the Group B was significantly higher ($p<0.05$) than Groups A and C on days 28 and 35 pi (Figure 17; Appendix 8).

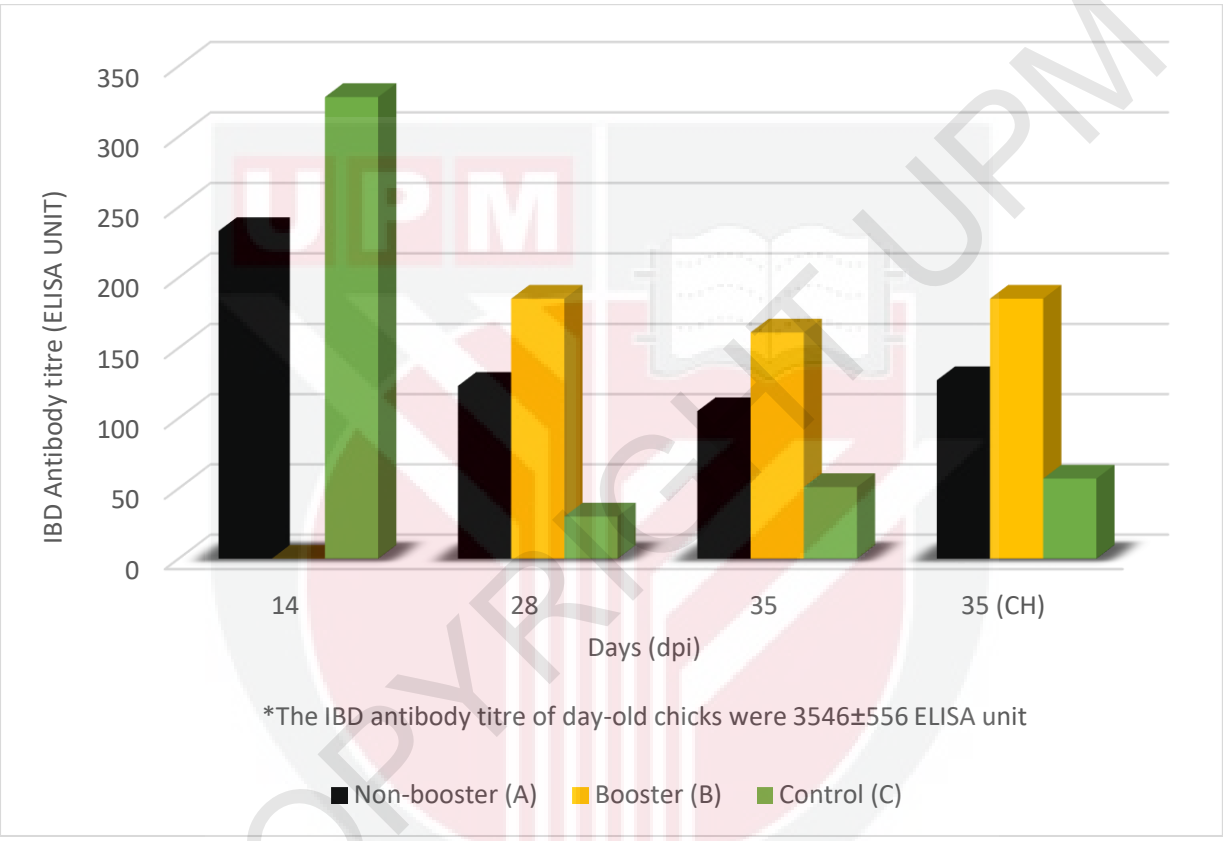

Figure 17: IBD antibody titer of chickens throughout the trial.

5.0 DISCUSSION

The safety of inactivated nVarIBDV was evaluated by nine parameters, which were the clinical signs, body weight, bursa weight, bursa to body weight ratio, gross lesions, histological lesions, bursa lesion scoring, virus loading and shedding, and IBD antibody titre. Throughout the trial, all the chickens in all groups were normal and healthy. No abnormal clinical signs were observed. This finding is consistent with previous studies which concluded that the nVarIBDV caused no obvious clinical symptoms and death (Fan et al., 2019).

According to Fan et al., (2019), chickens infected with the nVarIBDV had their body weight was negatively influenced. For all groups, the body weight increased steadily until day 35. Thus, it was concluded that the inactivated nVarIBDV did not interfere with the growth performance of the chickens as a pathogenic field isolate would have. Among the challenged chickens, Group B had the highest body weight than Groups A and C. The body weight of the challenged chickens in Groups A and C were lower than the non-challenged chickens. The body weight of challenged chickens in Group B was significantly higher than the non-challenged. This shows that immunization with a booster dose provides the highest level of humoral immune response against nVarIBDV by maintaining the growth performance of infected chickens.

The nVarIBDV damages the bursa of Fabricius and causes severe bursal atrophy (Fan et al., 2020; Thai et al., 2021; Myint et al., 2021). In this study, the bursa weight was not significantly different for all groups except on day 35 of the challenged chickens. Thus, it can be said that the inactivated nVarIBDV did not affect the bursa of chickens. The bursa weight of challenged chickens in Group B was significantly higher than Groups A and C, indicating that the booster dose provides the best level of protection to the bursa against the nVarIBDV field isolate.

A more accurate parameter to determine bursa atrophy caused by nVarIBDV is by referring to the bursa to body weight ratio. The justification for this is based on heavier chickens generally possessing heavier bursa which might be misleading in some cases. Overall, the bursa to

body weight ratio was not significantly different for all the groups except on day 35 among challenged chickens. The bursa to body weight ratio of challenged chickens in Group B was significantly higher than Groups A and C. This results is consistent with a study by Wang et al., (2020), in which the bursa to body weight index was significantly lower in the control group compared to the group that has received the live trial vaccine for nVarIBDV. This findings more accurately represents the efficacy of the inactivated nVarIBDV booster dose in preventing bursal atrophy in chickens infected with nVarIBDV.

According to a study by Fan et al., (2019), the bursa of chickens with nVarIBDV appeared to be atrophied, haemorrhages and yellowish with inflammatory exudation at 3-5 dpi. Similar gross lesions were identified in studies conducted by Thai et al., (2020) and Myint et al., (2020) although congestion of the bursa appeared to be an additional finding. In this study, no gross lesions were present in the bursa of chickens inoculated with inactivated nVarIBDV at 0, 14, 28 and 35 dpi. This indicates that the inactivated nVarIBDV does not cause gross pathological changes in the bursa and it can be safely used. The bursa of the challenged chickens in all groups also showed no gross lesions. The absence of lesions in the challenged Group C might be attributed to the low dose inoculation of the pathogenic field strain nVarIBDV and the reduced duration between the challenge virus inoculation and the bursa sampling. The latter may have not been sufficiently long enough to be able to elicit a clinical manifestation of a nVarIBDV in the bursa of Group C.

Typical histological lesions indicative of nVarIBDV are severe follicular lymphoid necrosis and depletion, multifocal follicular lymphoid infiltration with minimal to no inflammatory response. In addition, reticular and macrophages infiltration in lymphoid follicle, cystic cavities in lymphoid follicle, proliferation of fibrous tissues, severe follicle atrophy and infolding epithelium into damaged follicle have also been recorded (Fan et al., 2019; Myint et al., 2020, Thai et al., 2020). In this study, no significant histological lesions were recorded in chickens inoculated with inactivated nVarIBDV at days 0, 14, 28 and 35 (non-challenged) dpi. This suggests that the inactivated nVarIBDV does not cause histopathological changes in the bursa of chickens

and is therefore safe to be used. Among the challenged chickens, Group A and Group B showed no significant histological changes whereas Group C had mild to moderate degeneration and necrosis of lymphoid cells in the bursa follicles. Infiltration of inflammatory cells were also observed in the bursa of Group C. The results demonstrated in this study is in line with findings by Wang et al., (2021) where no microscopic lesions were observed in the vaccinated groups. The present results further confirms that the inactivated nVarIBDV is able to elicit a sufficient humoral immune response and prevent histopathological damages in the bursa of infected chickens.

The histological lesions of the bursa were scored to provide a better understanding and statistical picture of the bursa lesions. For all groups, the bursa lesion score was not significantly different throughout the trial. The bursa lesion score of challenged Group C was higher than Groups A and B although this findings were not statistically significant. Together with the above histological lesions results, the current findings confirm the efficacy of inactivated nVarIBDV in providing an immunoprotective effect on infected chickens.

Molecular virus detection and quantification in the bursa and cloacal swab samples were conducted using RT-qPCR to measure the virus loading and shedding. For both sample types, the virus copies of Group C was higher than Groups A and B. Because of the lack of previous studies pertaining to evaluating virus loading and shedding among inoculated chickens for nVarIBDV, it remains unclear to which degree the viral copies are attributed to improved efficacy of the trial vaccine. However, the results of the RT-qPCR finds clear evidence for the ability of the inactivated nVarIBDV to elicit the production of sufficient neutralizing antibodies that reduce the viral load and shedding among the infected chickens.

The study showed that the IBD antibody titer in the Group B was significantly higher than groups A and C at 28 and 35 dpi. The results indicated that serum antibodies of Group B induced by inactivated nVarIBDV could neutralize the field isolate nVarIBDV with average neutralizing titer of 185 ±18.37 and 161±17.69 ELISA units at 28 and 35 dpi, respectively. Humoral immunity plays a vital role in the immune protection of IBDV (Yang et al., 2020). It is

well-known that neutralizing antibody is a key component to control IBDV (Berg, 2000). Thus, it is shown here that the inactivated nVarIBDV as a potential vaccine candidate performs well and generates promising results on the antibody titer of chickens. There is no significant difference in the antibody titre of the challenged and non-challenged groups. This may be explained due to two limitations, firstly the shortened duration between the challenge virus inoculation and the sampling which may have not provided sufficient time for an immune response. Secondly, this may also be attributed to the lack of studies on the true pathogenicity and virulence status of nVarIBDV which may affect the degree of immune response elicited.

6.0 CONCLUSION

In conclusion, this study suggests that inactivated nVarIBDV has sufficient efficacy and safety to chickens immunized with a booster dose.

7.0 RECOMMENDATIONS

In future studies, it is recommended to use specific pathogen free (SPF) chicks instead of commercial broiler chickens to avoid maternal antibody interferences during the trial. Finally, the duration of the trial should be increased to better study the effects of inactivated nVarIBDV over time.

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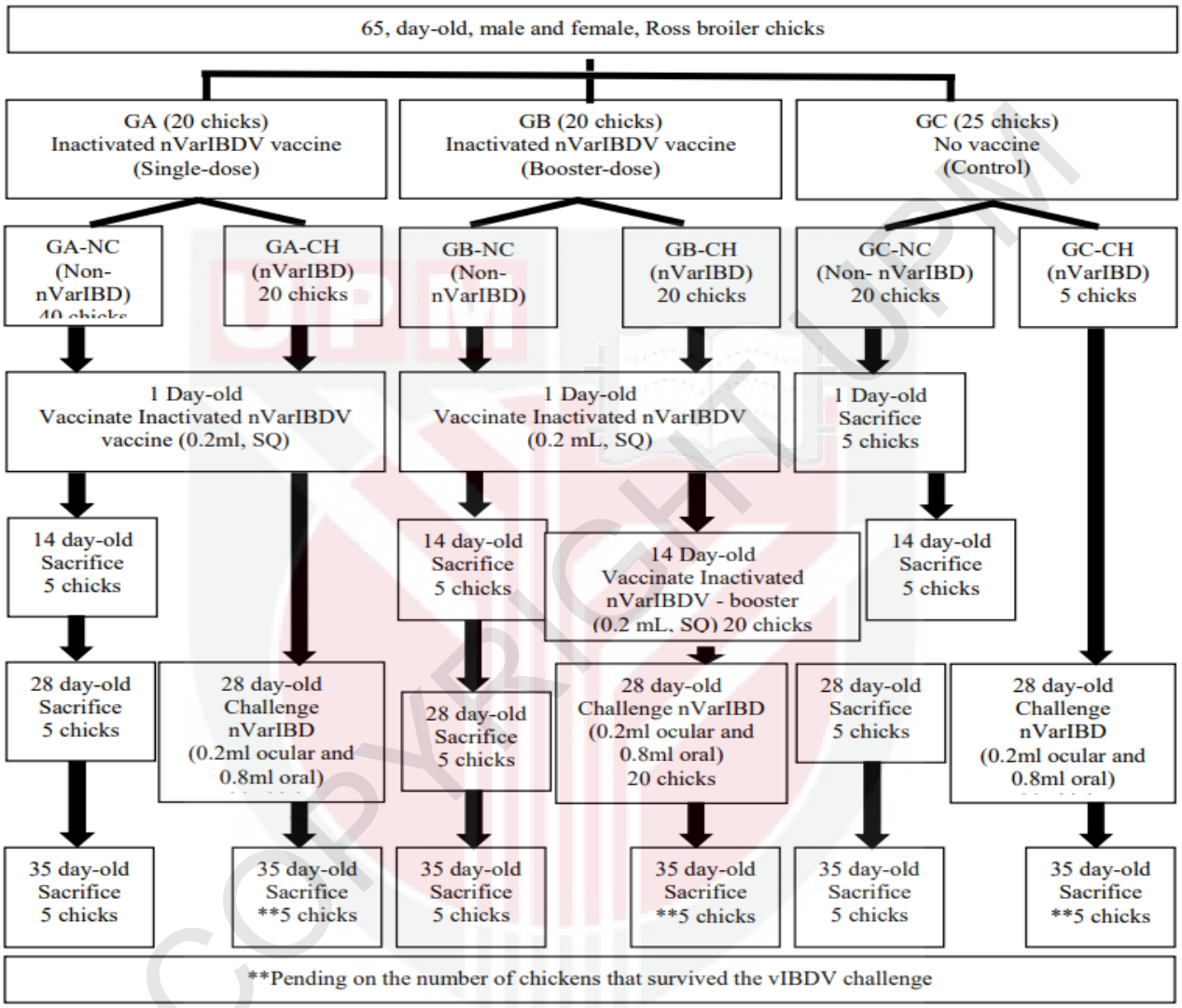
APPENDIX 1

Experimental design for efficacy and safety of inactivated novel variant infectious bursal disease virus in broiler chickens



After inoculation, all the chicks were monitored for any clinical signs and abnormalities twice daily. Feed and water were given *ad-libitum* throughout the trial. Prior to sacrificing, body weight was recorded and blood samples were taken to determine IBD antibody titre using ELISA technique. Necropsy was then conducted to examine the gross lesions. The bursa was collected, weighed and fixed in 10% buffered formalin for histological examination. PCR was done on bursa and cloacal swab samples.

APPENDIX 2

Histological lesion grading of the Bursa of Fabricius (Hair Bejo et al., 2000).

Lesion score	Description
0	No lesion, normal bursa
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.
4	Moderate to severe depletion of lymphoid cells in the follicles. The intra and extra follicular areas are hyperaemic and haemorrhagic. Thickened, corrugated and vacuolated epithelium in some areas.
5	Acute: Moderate to severe atrophy of bursal follicles, cellular degeneration and necrosis involving both cortex and medulla. Follicular cysts with fibrinous exudate and cell debris can be observed. Interstitial connective tissues were obvious, oedematous, and infiltrated with mild to moderate inflammatory cells. Chronic: Severe follicular atrophy, with cyst formation within the follicles and epithelial lining of organ. Remarkable infiltration of fibroblast in the interstitial area. Lymphocyte and monocyte infiltration commonly observed.

APPENDIX 3

Body weight of chickens throughout the trial

Groups	Body weight (g)				
	Days (pi)				
	0	**14**	**28**	**35**	**35 (CH)**
A (Non-booster)	-	568.5 ±47.2^{b}	2148.8 ±97.7^{c}	2705.4 ±82.3^{ab}	2264.2 ±74.1^{ac}
B (Booster)	-	-	1781 ±80.9^{c}	2522.8 ±84.6^{ab}	2543.8 ±173.3^{ac}
C (Control)	56.6 ±1.6^{a}	685.2 ±25.7^{b}	1788.2 ±91.3^{c}	2641.8 ±145.7^{ab}	2464.4 ±63.1^{ac}

Each value is the mean ± standard error of mean (SEM) of 5 chickens from each group. $^{a\text{-}b\text{-}c\text{-}ab\text{-}ac}$ means within column with no common superscripts differs at $p<0.05$.

APPENDIX 4

Bursa weight of chickens throughout the trial

Groups	Bursa weight (g)				
	Days (pi)				
	0	**14**	**28**	**35**	**35 (CH)**
A (Non-booster)	-	0.9450 ±0.1347^{b}	3.5025 ±0.2803^{c}	3.2540 ±0.4132^{ab}	1.7640 ±0.4483^{bc}
B (Booster)	-	-	2.7400 ±0.4446^{c}	2.9280 ±0.4227^{ab}	3.5100 ±0.2657^{a}
C (Control)	0.0925 ±0.006^{a}	1.2700 ±0.1691^{b}	2.5140 ±0.1168^{c}	3.6420 ±0.5365^{ab}	2.8860 ±0.2152^{bc}

Each value is the mean ± standard error of mean (SEM) of 5 chickens from each group. $^{a\text{-}b\text{-}c\text{-}ab\text{-}bc}$ means within column with no common superscripts differs at $p<0.05$.

APPENDIX 5

Bursa to body weight ratio of chickens throughout the trial

Groups	Bursa to body weight ratio ($x10^{-3}$)				
	0	**14**	**28**	**35**	**35 (CH)**
A (Non-booster)	-	1.6383 ±0.1127^{b}	1.6348 ±0.1251^{c}	1.2170 ±0.1850^{ab}	0.7908 ±0.2092^{bc}
B (Booster)	-	-	1.5193 ±0.1964^{c}	1.1450 ±0.1373^{ab}	1.4446 ±0.1865^{a}
C (Control)	1.6286 ±0.0649^{a}	1.8410 ±0.2296^{b}	1.4196 ±0.0962^{c}	1.3862 ±0.1975^{ab}	1.1656 ±0.0621^{bc}

Each value is the mean ± standard error of mean (SEM) of 5 chickens from each group. $^{a\text{-}b\text{-}c\text{-}ab\text{-}bc}$ means within column with no common superscripts differs at $p<0.05$.

APPENDIX 6

Bursa lesion score of chickens throughout the trial

Groups	Bursa lesion score				
	0	**14**	**28**	**35**	**35 (CH)**
A (Non-booster)	-	0.8000 ±0.2000^{b}	0.8000 ±0.2000^{c}	0.8000 ±0.3742^{ab}	1.0000 ±0.3162^{bc}
B (Booster)	-	-	0.4000 ±0.2449^{c}	1.000 ±0.3162^{ab}	1.2000 ±0.2000^{bc}
C (Control)	0.6000 ±0.2449^{a}	0.2000 ±0.2000^{b}	0.2000 ±0.2000^{c}	0.6000 ±0.2449^{ab}	1.4000 ±0.02449^{bc}

Each value is the mean ± standard error of mean (SEM) of 5 chickens from each group. $^{a\text{-}b\text{-}c\text{-}ab\text{-}bc}$ means within column with no common superscripts differs at $p<0.05$.

APPENDIX 7

Virus loading and shedding of challenged chickens day 35 pi

Groups	Log_{10} Observed Reaction (Copies/titer)	
	Bursa sample	**Cloacal swab sample**
A (Non-booster)	7.564	7.551
B (Booster)	7.057	7.154
C (Control)	9.563	8.205

APPENDIX 8

IBD antibody titre of chickens throughout the trial.

Groups	IBD Antibody Titre (ELISA unit)				
	Days (pi)				
	0	**14**	**28**	**35**	**35 (CH)**
A (Non-booster)	-	233 ±20^{b}	123 ±33^{c}	105 ±16^{ac}	127 ±26^{bc}
B (Booster)	-	-	185 ±18^{ab}	161 ±18^{a}	185 ±31^{bc}
C (Control)	3546 ±556^{a}	328 ±127^{b}	30 ±5^{c}	51 ±12^{ac}	57 ±7^{bc}

Each value is the mean ± standard error of mean (SEM) of 5 chickens from each group. $^{a\text{-}b\text{-}c\text{-}ab\text{-}ac\text{-}bc}$ means within column with no common superscripts differs at $p<0.05$.