UNIVERSITI PUTRA MALAYSIA

EFFECT OF DIFFERENT PASSAGES, TIMES AND ROUTES OF INOCULATION ON HUMORAL IMMUNE RESPONSE INDUCED BY ATTENUATED FOWL ADENOVIRUS IN BROILER CHICKENS

LEE SHIN JYE

Ip
FPV 2020 13

EFFECT OF DIFFERENT PASSAGES, TIMES AND ROUTES OF INOCULATION ON HUMORAL IMMUNE RESPONSE INDUCED BY ATTENUATED FOWL ADENOVIRUS IN BROILER CHICKENS

LEE SHIN JYE

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan.

OCTOBER 2020

It is hereby certified that I have read this project paper entitled "Effect of Different Passages, Times and Routes of Inoculation on Humoral Immune Response Induced by Attenuated Fowl Adenovirus in Broiler Chickens" by Lee Shin Jye and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

PROFESSOR DATO' DR. MOHD HAIR BIN BEJO

DVM (UPM), PhD (Livepool)

Professor

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

DR NORFITRIAH BINTI MOHAMED SOHAIMI

DVM (UPM), PhD (UPM)

Senior Lecturer

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude and deep regards to my respectable supervisor, Professor Dato' Dr. Mohd Hair bin Bejo and co-supervisor, Dr. Norfitriah binti Mohamed Sohaimi for their abundance professional suggestions and guidance throughout the planning and development of the project.

Next, I express my profound thanks to Dr. Ugwu, Mr. Sapuizaman and Ms. Nurhanis for their constant technical advices and assistance during all the laboratory work in Serology Laboratory. Not to forget all the staffs of Animal Research Facility for their help in taking care of chickens.

Besides, I also want to thank Dr. Shanmugavelu for his guidance in statistical analysis. A lot thanks to my final year project mates; Sofiah Hanim and Muhammad Irfan for their endless support and caring throughout the journey.

Last but not least, I would like to thank my beloved family and friends for motivating me throughout the project. It would have been not possible without the constant support and love from them.

Thank you.

CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

FAdV	Fowl Adenovirus
P15	Passage 15
P20	Passage 20
pi	Post inoculation
IBH	Inclusion Body Hepatitis
PCR	Polymerase Chain Reaction
ELISA	Enzyme-Linked Immunosorbent Assay
HE	Hematoxylin and Eosin
CEL	Chicken Embryo Liver
SPF	Specific Pathogen Free

ABSTRAK

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

KESAN DARIPADA PERBEZAAN LALUAN, MASA, AND CARA INOKULASI FOWL ADENOVIRUS YANG DILEMAHKAN TERHADAP TINDAK BALAS IMUN HUMORAL DI KALANGAN AYAM PEDAGING KOMERSIAL

Oleh

Lee Shin Jye

2020

Penyelia: Profesor Dato' Dr. Mohd Hair bin Bejo

Penyelia Bersama: Dr Norfitriah binti Mohamed Sohaimi

Fowl adenovirus (FAdV) membawa wabak penyakit and kerugian ekonomi yang teruk kepada industri ayam. Tujuan kajian ini adalah untuk menentukan kesan daripada perbezaan laluan, masa and cara inokulasi FAdV isolate (UPM1142) yang dilemahkan terhadap tindak balas imun humoral di kalangan ayam pedaging komersial. Tujuh puluh enam ekor anak ayam berusia sehari telah dibahagikan kepada lima kumpulan, iaitu kumpulan A, B, C, D dan E. Kumpulan A, B, C dan D terdiri daripada enam belas anak ayam setiap satu dan dibahagikan kepada tiga subkumpulan, iaitu kumpulan A1, B1, C1 dan D1; A2, B2, C2 dan D2; A3, B3, C3 dan D3. Kumpulan A dan C telah diinokulasi dengan FAdV isolate UPM1142 (1 x $10^{6.5}$ $TCID_{50}$ /mL) pasaj 20 (P20) yang dilemahkan masing-masing melalui cara bawah kulit and mulut. Kumpulan B dan D telah diinokulasi dengan FAdV isolat UPM1142 (1 x $10^{5.7}$ $TCID_{50}$ /mL) pasaj 15 (P15) yang dilemahkan masing-masing melalui cara bawah kulit and mulut.

Manakala kumpulan E bertindak sebagai kumpulan kawalan tanpa sebarang inokulasi. Makanan dan minuman disediakan secara *ad-libitum* dan sebarang perubahan klinikal juga diperhatikan secara dua kali sehari. Pada hari pertama, anak ayam dari kumpulan A1, B1, C1, D1, A2, B2, C2 dan D2 telah diinokulasikan sebagaimana telah ditetapkan. Empat ekor anak ayam dari kumpulan E dikorbankan melalui dislokasi servikal untuk pengambilan sampel. Berat badan dan sampel darah diambil sebelum nekropsi. Lesi matakasar dan berat hati direkodkan semasa nekropsi dijalankan. Sampel hati diambil untuk pemeriksaaan histologi dan diawat dalam 10% formalin buffered sementara titer antibodi FAdV dikesan dari sampel darah menggunakan teknik ELISA. Pada hari ke 7 selepas inokulasi, empat ekor anak ayam dari kumpulan A1, B1, C1, D1 dan E telah dikorbankan untuk pengambilam sampel. Manakala kumpulan A2, B2, C2, D2, A3, B3, C3, D3 telah diinokulasikan sebagaimana ditetapkan. Pada hari ke 21 selepas inokulasi, semua ayam telah dikorbankan untuk pengambilan sampel. Kajian ini menunjukkan bahawa tiada tanda klinikal, lesi matakasar dan histologi dalam semua ayam sepajang tempoh kajian. Ayam dalam semua kumpulan tidak menunjukkan perbezaan yang signifikan ($p>0.05$) dalam berat badan kecuali kumpulan E mempunyai berat badan yang paling rendah (964±23.51) berbanding dengan kumpulan C1, C2, C3, D1, D2, D3 pada hari ke 21 selepas inokulasi. Ayam dalam semua kumpulan tidak menunjukkan perbezaan yang signifikan ($p>0.05$) antara berat hati dan hati kepada nisbah berat. Keputusan ELISA menunjukkan kumpulan C1 terdapat titer antibodi paling tinggi (1557±528) pada hari ke 7 selepas inokulasi berbanding dengan kumpulan A1 (380±99), B1 (771±289), D1 (895±506) dan E (1256±730). Kumpulan B2 diinokulasi dua kali melalui cara bawah kulit dengan FAdV (P15) yang dilemahkan terdapat titer antibodi tertinggi (1956±916) berbanding

dengan kumpulan lain pada hari ke 21 selepas inokulasi. Namun begitu, titer antibodi FAdV tidak menunjukkan perbezaan yang signifikan ($p>0.05$) di antara kumpulan. Kesimpulannya, kajian ini mencadangkan bahawa FAdV (P15) yang dilemahkan diinokulasi dua kali melalui cara bawah kulit pada hari pertama and hari ke 7 umur ayam dapat memberi respon antibodi yang tertinggi jika berbanding dengan kumpulan lain. Di samping itu, FAdV yang dilemahkan adalah selamat diguna untuk ayam pedaging komersial.

Kata kunci: Fowl adenovirus yang dilemahkan, tindak balas imun humoral, masa dan cara inokulasi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfilment of the course VPD 4999 – Final Year Project.

EFFECT OF DIFFERENT PASSAGES, TIMES AND ROUTES OF INOCULATION ON HUMORAL IMMUNE RESPONSE INDUCED BY ATTENUATED FOWL ADENOVIRUS IN BROILER CHICKENS

By

Lee Shin Jye

2020

Supervisor: Professor Dato' Dr Mohd Hair bin Bejo

Co-supervisor: Dr Norfitriah binti Mohamed Sohaimi

Fowl adenovirus (FAdV) infection may lead to disease outbreak and huge economy loss in the poultry industry. The objectives of the study were to determine the effect of different passages, times and routes of inoculation on humoral immune responses induced by attenuated FAdV isolate (UPM1142) in broiler chickens. Seventy-six, day-old chicks were divided into five groups, namely Groups A, B, C, D and E. The Groups A, B, C and D each consisted of 16 chicks each and further divided into 3 subgroups namely Groups A1, B1, C1 and D1; A2, B2, C2 and D2, and A3, B3, C3 and D3. The Groups A and C were inoculated with attenuated FAdV UPM1142 isolate (1 x $10^{6.5}$ $TCID_{50}$ per mL) passaged 20 (P20) subcutaneously and orally, respectively. The Groups B and D were inoculated with the attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per mL) passaged 15 (P15) subcutaneously and orally, respectively, while Group E acted as control group and remain un-inoculated. The chickens were given feed and water *ad-*

libitum and monitored for any abnormal clinical signs twice daily. On one day of age or day 0 post-inoculation (pi), chickens in Groups A1, B1, C1, D1, A2, B2, C2 and D2 were inoculated with the virus accordingly. Four chicks from Group E were sacrificed by cervical dislocation for sampling. Body weight and blood samples were taken prior to necropsy. Gross lesion of liver and liver weight were recorded during necropsy. The liver samples were collected and fixed in 10% buffered formalin for histological examination, while FAdV antibody titer was detected from the blood samples using ELISA techniques. On day 7 pi, four chicks from Groups A1, B1, C1, D1 and E were sacrificed for sampling, while Groups A2, B2, C2, D2, A3, B3, C3 and D3 were inoculated with the virus accordingly. On day 21 pi, all the rest of the chickens were sacrificed for sampling. This study revealed that neither abnormal clinical signs nor gross and histological lesions were recorded throughout the trial. There was no significant difference ($p>0.05$) in body weight in all groups of chickens, except the Group E recorded the lowest ($p<0.05$) body weight (964±23.51) compared to the Groups C1, C2, C3, D1, D2 and D3 on day 21 pi. The chickens in all groups showed no significant differences ($p>0.05$) in their liver weight and liver to body weight ratio. The Group C1 had the highest antibody titer (1557±528) compared to groups A1 (380±99), B1 (771±289), D1 (895±506) and E (1256±730) on day 7 pi. On day 21pi, Group B2 that inoculated twice with P15 attenuated FAdV subcutaneously had the highest antibody titer (1956±916) among other groups. Overall the FAdV antibody titer induced showed no significant differences ($p>0.05$) across all groups. In conclusion, the study suggests that attenuated FAdV at passage 15 and inoculated twice subcutaneously at day old and 7 of age able to induce higher antibody

response when compared to the other groups. Attenuated FAdV is safe to use in commercial broiler chickens.

Keywords: Attenuated fowl adenovirus, humoral immune response, time and route of inoculation.

1.0 INTRODUCTION

1.1 Background of the Study

Fowl adenovirus (FAdV) is classified under the genus Aviadenovirus in the adenovirus family (Hess, 2013). Fowl adenoviruses (FAdVs) are grouped into five species (A-E) based on their molecular structure and subsequently divided into 12 serotypes (1-7, 8a, 8b, 9-11). FAdV infection in poultry will cause diseases such as inclusion body hepatitis (IBH), hydropericardium syndrome, quail bronchitis, turkey haemorrhagic enteritis and marble spleen disease (McFerran and Smyth, 2000). Adenovirus infection has consequences on food conversion and growth. Adenovirus infection will cause decreased food consumption. The affected birds may also has decreased body weight or even causing high mortality (Adair and Fitzgerald, 2008).

Inclusion body hepatitis was first discovered in USA in 1963 (Helmboldt and Frazier, 1963). Adenovirus-induced IBH also occurred in other avian species such as turkeys, geese, pheasants and psittacines (Cowen, 1992). Inclusion body hepatitis was first reported in Malaysia in year 2005 by Hair-Bejo in a commercial farm in Perak, which the broilers shown poor growth and high mortality of 10%. The liver shown mild to moderate enlarged with pale, friable and fatty changes appearance and areas of haemorrhages and congestion upon necropsy. The kidney was also shown pale and slightly enlarged. Numerous eosinophilic and basophilic, round or irregularly shaped intranuclear inclusion bodies were found in the hepatocytes upon histological examination of the liver. The hepatic parenchyma was moderately degenerated and necrotized. The liver also shown moderate congestion with areas of haemorrhages and moderate to severe infiltration of mononuclear inflammatory cells (Hair-Bejo, 2005).

Attenuated virus vaccine is created by passaging a virus in cultured cells and has proven to be an effective measure in preventing many viral diseases (Lauring et al.,2010). It was reported that FAdV with lower passage has higher virus titer than higher passage (Sohaimi et al., 2019). The virus virulence was decreased at high passage with delayed mortality in embryos (Sohaimi et al., 2018). There were no significant different between route of inoculation either via oral or subcutaneous route in term of induction of the antibody titre (Sohaimi et al., 2018). The incidence of carriage and symptomatic disease can be reduced by booster dose (Charania and Moghadas, 2017).

Currently, there is minimum data showing the effect of passage, times and routes of inoculation of attenuated FAdV on humoral immune response induced by the virus in broiler chickens. The use of optimum passage and route of administration with or without booster are important to be determined for vaccine production and vaccination programme.

1.2 Hypothesis

The hypothesis of the study were:

1. The number of passages of attenuated FAdV effects humoral immune response.
2. The time of inoculation of attenuated FAdV effects humoral immune response.
3. The route of inoculation of attenuated FAdV effects humoral immune response.

4. The number of passages of attenuated FAdV does not affect humoral immune response.

5. The time of inoculation of attenuated FAdV does not affect humoral immune response.

6. The route of inoculation of attenuated FAdV does not affect humoral immune response.

1.3 Objectives

The objectives of the study were to determine:

1. The effect of different FAdV passages in chicken embryo liver (CEL) cells on the induction of humoral immune response in broiler chickens.
2. The effect of different time of the FAdV inoculation on the induction of humoral immune response in broiler chickens.
3. The effect of different routes of the FAdV inoculation on the induction of humoral immune response in broiler chickens.
4. The safety and immunogenicity of the FAdV isolate.

2.0 LITERATURE REVIEW

2.1 Aetiology

2.1.1 Taxonomy

Adenovirus comprised of five genera which are Mastadenovirus, Aviadenovirus, Siadenovirus, Atadenovirus and Ichtdenovirus (Hess, 2013). Fowl adenovirus (FAdV) is classified under the genus Aviadenovirus in the adenovirus family. FAdVs are grouped into five species (A-E) based on their molecular structure and subsequently divided into 12 serotypes (1-7, 8a, 8b, 9-11) (Hess, 2013). FAdV comprised of three group namely group I, II and III. Group I FAdV is responsible for inclusion body hepatitis (IBH), hydropericardium syndrome (HPS) and quail bronchitis. Group II FAdV is responsible for turkey haemorrhagic enteritis and marble spleen disease while Group III FAdV is responsible for egg drop syndrome (McFerran and Smyth, 2000).

2.1.2 Morphology

Adenovirus is icosahedral, non-enveloped double stranded DNA virus that has size of 74 – 90nm. The virion consists of 252 capsomers and surrounded by a core 60 – 65nm in diameter. Capsomers are arranged in triangular faces around each edge, with six capsomers (Hess, 2013). The major proteins of the FAdVs capsid are hexon, penton base and fibre (Niczyporuk, 2018). Pentons are the capsomeres that found at the vertices whereas hexons are the non-vertex capsomeres (Mark et al., 2007). There are 240 non-vertex capsomers (hexon) with a diameter of 8-9.5 nm, and 12 capsomers

(penton bases) (Hess, 2013). There are two pin-shaped projection at each vertex which called fibre (Mark et al., 2007).

Hexon is the most abundant viral surface protein and it harbors the main neutralizing epitope that can be seed for serotyping through neutralization tests. Penton involves in internalization of the virus. Fiber facilitates the binding of virus particle to the host cell surface (Uusikerttula et al, 2015).

2.2 Transmission

FAdV is transmitted vertically and horizontally (Chen et al, 2019). Major route of transmission is vertical route. FAdV transmitted through embryonated eggs (Adair and Fitzgerald, 2008). Viral antigens can be found in the embryonic egg in egg yolk and the albumen and virus are frequently reactivated in cell cultures produced from embryos and young chicks taken from infected flocks (Scott, 2017). At the time of hatching, the chicks that hatched from the infected eggs may excrete virus in the feces (McFerran and Smyth, 2000). Even though adenoviruses can be isolated from day 1 post-infection, but viruses usually excreted after 3 weeks. Peak excretion occurred in broiler at age of two to four weeks while at five to nine weeks post-infection in layer (Adair and Fitzgerald, 2008). Presumably, the latent virus will reactivate after the maternal antibody declined (McFerran and Smyth, 2000). Virus can also be reactivated in few weeks old chicks especially those are immunosuppressed (Helena et al, 2006).

Fowl adenovirus transmitted horizontally via feces, aerosol, fomites, transport and personnel as they present in all excretion (Scott, 2017; Helena, 2006). The virus can spread through all excretions as it presents in feces, tracheal and nasal mucosa, intestines and cecal tonsils. However, it is high titers in feces where it can survive for weeks. The virus may also be found in the semen, posing a potential risk when using artificial insemination (Hess, 2013).

2.3 Pathogenicity

Pathogenicity of FAdV infection is affected by the serotypes and genotypes involved (McFerran and Adair, 1977). The ability to produce illness, respiratory disease or even death are varied in different serotypes or in strains of the same serotype (Adair and Fitzgerald, 2008).

The role of FAdV as primary pathogens is not clearly established (Adair and Fitzgerald, 2008). Species A strain FAdV-1 caused gizzard erosion and ulceration. Inclusion body hepatitis (IBH) usually associated with species D (FAdV-2 and FAdV-11) and E (FAdV-8a and FAdV-8b). Hydropericardium syndrome (HPS) caused by species C strain FAdV-4. IBH and HPS are highly pathogenic to chickens (Cizmecigil et al, 2020). Hydropericardium syndrome has higher mortality than IBH as the disease is characterized by accumulation of fluid in the pericardial sac (Brown et al, 2018).

Maternal antibodies to the homologous strain may restrict the spread of virus and protection of that serotype infection may present, preventing reinfection of the same serotype for some time. Multiplication of virus within the host and mortality it caused

decreased with increased age (McFerran and Adair, 1977). Inclusion body hepatitis or HPS outbreaks in older chickens most likely involve concurrent marked immunosuppression (Toro et al, 2000). Coinfection with infectious bursal disease virus (IBDV) or chicken infectious anemia virus (CIAV) will enhance the pathogenicity of FAdV (Hess, 2013).

The virus entered the host cell and transferred virus DNA to the nucleus. Transcription and translation of the so-called early (E) genes were carried out. The early gene redirect cellular function coded the proteins to facilitate the replication of the virus DNA while the transcription and translation of the late (L) genes coded for the virus structural proteins. After disruption of the nuclear membrane and released of virus by cell destruction, the viral proteins assembled into complete virions in the nucleus (Hess, 2013).

2.4 Clinical Signs

Inclusion body hepatitis is typically seen in meat-producing birds between the age of three and seven weeks, but can be as young as seven days and as old as twenty weeks. Inclusion body hepatitis associated with sudden onset of mortality that peak within three to four days and halts by five to six days, while deaths persisted for up to three weeks in some outbreaks (McFerran and Smyth, 2000). The morbidity is low. Mortality may reach 10% and occasionally go up to 30% (Hess, 2013). There were outbreaks of IBH in chickens less than 3 weeks-old in Australia with mortality up to 30% (Adair and Fitzgerald, 2008). The birds infected with IBH may presented with

crouching position with ruffled feathers, die within 48 hours or recover. Higher mortality in younger birds that less than three weeks old (Hess, 2019).

2.5 Pathology

2.5.1 Gross Lesions

Inclusion body hepatitis primarily affecting the liver. Pale, friable, swollen liver are the main lesion of inclusion body hepatitis. Petechial or ecchymotic hemorrhages may be present in the liver and skeleton muscle (Adair and Fitzgerald, 2008). Presence of small white foci on the liver and may involve petechial or haemorrhages. Swollen kidneys usually coincide with glomerulonephritis. Other lesions such as atrophy of the bursa and thymus, aplastic bone marrow and hepatitis are included (Hess, 2019).

2.5.2 Histological Lesions

On the histological examination of liver, there is presence of inclusion bodies in the hepatocytes. The inclusion bodies can be eosinophilic with large, round or irregular shaped with a clear pale halo or occasionally basophilic. Basophilic inclusion bodies are more prominent in the cases of IBH in Australia (Adair and Fitzgerald, 2008). Hair-Bejo (2005) reported that the eosinophilic inclusion bodies contain only fibrillary granular material and filaments while the basophilic intranuclear inclusion bodies contain numerous adenoviruses when observed under transmission electron microscopy. The presence of eosinophilic inclusion bodies in hepatocytes indicates

early stage of formation of virus or late stage after the virus has left the nucleus (Norina et al, 2016).

2.6 Diagnosis

The diagnosis of adenovirus infection can be classified into direct and indirect method. The direct methods are virus detected through electron microscope, immunofluorescence test, polymerase chain reaction (PCR), viral neutralization test, and histopathology. The indirect method is serology which is detection of antibodies (Hafez, 2011).

2.6.1 Virus Isolation and Identification

Feces or colon with feces is the preferred sample. If the particular organ has obvious lesion is also needed such as liver in IBH cases. A 10% suspension of the specimen in cell culture media or bacteriological broth is prepared. The isolation of virus usually carried out in cell cultures, the FAdV ideally isolated in chick embryo liver or chick kidney cells (McFerran and Smyth, 2000). The confirmation of the FAdV infection can be carried out by electron microscope due to its characteristic morphology (Hess, 1999). Immunofluorescence test can be used to detect antigen or virus particles (Hafez, 2011).

Polymerase chain reaction is primary assay for detection of FAdV. The assay are high sensitivity, simplicity, selectivity and rapidity (Li et al, 2017). Polymerase chain reaction coupled with DNA sequencing or enzyme restriction analysis, was most

effective in the identification and differentiation of some or all of the 12 FAdV serotypes. However, it is time consuming and often require extensive analysis and can be relatively costly to use as a routine typing tool (Steer, 2009).

Viral neutralization test is used to determine the serotype of a virus that has been isolated. The virus neutralization test with the isolate against standard reference antiserums must be applied to all known serotypes (Scott, 2017). Histopathology can be used for identification and detection of intranuclear inclusion bodies in hepatocytes (Hafez, 2011). A non-specific indication of the existence of DNA-containing virus is provided by hematoxylin and eosin (HE) staining of infected cell monolayers or tissue parts and intranuclear basophilic inclusions (Hess, 2013).

2.6.2 Serology

Recently, FAdV serological techniques have focused mainly on various modifications of enzyme-linked immunosorbent assay (ELISA). In earlier versions, an indirect ELISA with whole viruses as coating antigens was employed to detect antibodies to FAdV in tissue samples from chickens that undergoing natural and experimental infections (Dawson et al, 1980). The assay is the test of choice as it can detect group antibody in commercial birds and given estimation of extend of infection (McFerran and Smyth, 2000).

Immunodiffusion test is the most common serologic test for detection of group specific antigen. However, it is lack of sensitivity (Hafez, 2011). The birds with primary infection due to natural exposure may not react with precipitin antibodies (McFerran

and Smyth, 2000). Serum neutralization test can be used to detect the type-specific antibody but it is time-consuming and costly (McFerran and Smyth, 2000).

2.7 Control and prevention

Biosecurity practices is essential to prevent adenovirus infection. Proper management, cleaning and disinfection of premises and equipment, restricted visitor entry, and vaccination crews in poultry houses play an important role in disease prevention (Hafez, 2011). Used of IBD vaccine has proven beneficial in preventing FAdV as the IBD passive immunity is essential to reduce the effect of young age FAdV infection in broiler chicks (Cowen, 1992).

Live vaccines induced innate and adaptive immune responses, while inactivated vaccines are most widely used after vaccination of live vaccine in layer and breeder chickens to boost immunity against many diseases and provide protection in progeny through transfer of maternal antibodies (Ekanayake, 2009). In Australia, monovalent live FAdV-8b vaccine was used in broiler breeder vaccination programs to protect broilers from IBH caused by FAdV-8b (Gupta, 2018). Vaccination of replacement breeders in mid-rearing by using live, virulent FAdV-8b vaccine able to prevent acute IBH outbreaks in progeny (Ekanayake, 2009).

Although the vaccine is used, the spread of FAdV is still possible due to inappropriate inactivation of virulent strains, poor hygienic conditions during vaccine development, lack of a suitable adjuvant and appropriate quantity of virus (Li et al, 2017).

2.8 Effect of Fowl Adenovirus on Immune System

Immune response toward FAdV is species and serotype specific (Adair and Fitzgerald, 2008). Virulent FAdV-1, FAdV-4 and FAdV-8 infections shown a possible influence of the immune system due to pathogenicity of these viruses. Virulent FAdV-4 strains shown a predilection for lymphoid tissues from several reports (Schonewille et al, 2008). Saifuddin and Wilks (1992) reported that FAdV-8 field strain shown occurrence of a severe lymphocytic depletion in the bursa of Fabricius, thymus and spleen in specified pathogen-free (SPF) chickens following infection. There were severe IBH outbreaks in New Zealand that caused mortality up to 30% in several broiler flocks which associated with lymphocytic depletion in the bursa and thymus. These outbreaks were not associated with IBH virus infection or may other identifiable immunosuppressive factors (Saifuddin and Wilks, 1992). Hence, it is believed that the virulent FAdV can cause decrease of CD3+, CD4+ and CD8+ T-lymphocytes in the spleen and thymus, suggestive there is presence of immunosuppressive ability (Schonewille et al., 2008).

3.0 MATERIALS AND METHODS

3.1 Ethics Statement

This study was approved by the Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM/IACUC/AUP-U036/2020), and it was performed in accordance with the “Animal Act 1953 (Revised 2006)” and “Wildlife Conservation Act 2010”. All the chickens were cared for in accordance with humane procedures.

3.2 Attenuated FAdV

The attenuated FAdV isolates namely UPM11142 passage 15 (P15) and 20 (P20) with virus titre of $10^{5.7}$ $TCID_{50}$ per 0.1 mL and $10^{6.5}$ $TCID_{50}$ per 0.1 mL, respectively were obtained from Professor Dato' Dr Mohd Hair bin Bejo, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The FAdV isolate was prepared through passaging of the virus into primary chicken embryo liver (CEL) cells for 15 (P15) and 20 (P20) times passages. Briefly, the liver from infected chicken was collected and processed, prior treated with commercial antibiotic-antimycotic solution in 1 in 10 (v/v) dilution. Then, the liver homogenate was incubated at 4°C for 1 hour prior inoculation. The virus inoculum (0.1mL) was inoculated into SPF chicken embryonated eggs (CEE) via chorioallantoic membrane (CAM) route. The liver of embryos was harvested for the next passage in SPF eggs. Homogenate liver embryos from passage 2 was prepared for inoculation into primary CEL cells. The primary CEL cells was prepared from the SPF CEE for passaging of FAdV isolate up to 20 consecutive passages. Subsequently, viral supernatant from passage 15 and 20 were used in this study. FAdV isolate from P15 and P20 adapted in primary CEL cells was molecularly characterized in hexon and fiber genes. Several molecular changes were detected in both proteins indicates presence of adaptation and attenuation genes (Sohaimi et al., 2019).

3.3 Commercial Broiler Chicken

Day-old commercial broiler chicks were obtained from the Linggi Poultry Farm (M) Sdn. Bhd., Rembau, Negeri Sembilan. The chicks were housed in the Animal Research

Facilities, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang throughout the 21 days of study.

3.4 Experimental Design

A total of 76-day-old commercial broilers chicks were divided randomly into five groups namely Groups A, B, C, D and E. The Groups A, B, C and D each consisted of 16 chicks. These group further divided into 3 subgroups namely Groups A1, B1, C1 and D1; A2, B2, C2 and D2, and A3, B3, C3 and D3. The Groups A and B were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) subcutaneously (0.2mL) and orally (0.5mL), respectively. The Groups C and D were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) subcutaneously (0.2mL) and orally (0.5mL), respectively. Group E consisted of 12 chicks, which served control group and remained uninoculated. On Day 1 of age, four chicks from control group (Group E) were sacrificed by cervical dislocation for first sampling. The Groups A1, B1, C1 and D1 of eight chicks in each group and Group A2, B2, C2 and D2 of four chicks in each group were inoculated with the attenuated FAdV at day old (day 0 post inoculation (pi)). The chicks were given feed and water *ab-libitum* and monitored the clinical signs twice daily. On day 7 post inoculation (pi), four chicks from Group A1, B1, C1, D1 and E were sacrificed by cervical dislocation for sampling. Chicks from Group A2, B2, C2, D2, A3, B3, C3 and D3 of four chicks in each group were inoculated with the attenuated FAdV. On day 21 post pi, the remaining four chicks from each group were sacrificed for last sampling. Prior to necropsy, the body weight was recorded and blood sample were collected to determine

the FAdV antibodies titre by using enzyme linked immunosorbent assay (ELISA) technique. The necropsy was then conducted to examine the gross lesions. The liver was collected, weighed and fixed in 10% buffered formalin for histological examination (Appendix 1).

3.5 Histopathology

The liver was fixed in 10% buffered formalin for at least 24 hours. After that, the samples were trimmed at size of 1cm × 1cm × 0.3cm and put in the cassettes to be processed in the automatic tissue processor (Leica) for at least 16 hours. The samples were dehydrated in a series of alcohol and cleared with xylene in the automated tissue processor. Then, the samples were embedded in paraffin wax and cooled to be harden. The samples were then sectioned into thin slide of 3-4µm using microtome (Leica). The tissue ribbons were transferred into a warm water bath at around 45°C carefully after sectioned. The floating sectioned tissue were scooped and mounted into the glass slide. The glass slides were then placed on the slide warmer (Leica) at 50°C for 30 minutes to melt the excess paraffin wax. Lastly, the slides were stained with Hematoxylin and Eosin (HE). The slides were soaked into xylene, 70%, 90% and 100% alcohol before and after staining with HE. After staining, the slides were mounted with cover slips by DPX and dried. The slides were observed under microscope using 4X, 10X, 20X and 40X objective lens for any histological changes (Fischer et al., 2008).

3.6 Enzyme-linked Immunosorbent Assay

Serum samples were collected from the blood sample and tested for FAdV antibody titre using ELISA technique. The ELISA test was carried out at Institute Bioscience, Universiti Putra Malaysia using Fowl Adenovirus Group I Antibody test kit (BioCheck). Firstly, the serum samples were diluted into 1:1000 in the sample diluent reagent in the dilution plate. Then, 100µL of positive and negative control were added into the respective wells of the microtiter plates that pre-coated with FAdV antigen. After that, 100µL of diluted samples were added into the appropriate wells. The plate was then covered with lid and incubated at room temperature for 30 minutes. Next, 350µL of wash buffer was used to wash the wells for at least four times. After washed, the plate was inverted and tapped firmly on the absorbent paper until no moisture was visible. Then, 100µL conjugate reagent was added into the wells and covered with the lid again to be incubated in room temperature for another 30 minutes. The wash procedure was repeated. Substrate reagent (100µL) was added into the wells and incubated for another 15 minutes. Lastly, the 100µL of stop solution was added into the appropriate wells to stop the reaction. After 15–20 minutes, the result of ELISA plate was read by using microtiter plate reader (BioTeK). The FAdV antibody titre results were generated by the BioChek 2000 software.

3.7 Statistical Analysis

The data for body weight, liver weight, liver to body weight ratio and FAdV antibody titre of the chickens were analyzed by using IBM SPSS Statistics version 25. Parametric test (1-way analysis of variance (ANOVA)) was used to analyze the data, where $p<0.05$ indicated that there was significant difference between the groups.

4.0 Results

4.1 Clinical Signs

All chickens in all groups were actively feeding and drinking normally with no abnormal clinical signs and mortality observed throughout the study (Figure 1).

Figure 1: Normal conditions of chickens throughout the trial. (a). Day 0 pi (Group A1). (b). Day 7 pi (Group B2) and (c). Day 21 pi (Group C3).

4.2 Body Weight

Body weight of chickens in all groups increased significantly from day 0 to day 21 pi. The body weight of the day-old chicks (DOC) of Group E was 67.4±3.3g on day 0 pi and it increased to 250.0±13.0g and 964.0±23.5g on day 7 and day 21 pi respectively. On the day 7pi, the body weight of the chickens in Groups A1, B1, C1, D1 and E were 261.8±16.4g, 251.5±22.7g, 279.0±8.5g, 243.3±20.1g and 250.0±13.0g respectively. Group C1 had the highest body weight (279.0±8.5g) on day 7 pi. On day 21 pi, the body weight of chickens in Groups A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3 and E were 1096.5±38.6g, 1116.3±58.1g, 1102.5±27.8g, 1030.8±20.5g, 1071.8±48.5g, 1110.5±21.2g, 1411.8±43.8g, 1276.3±35.5g, 1244.5±41.5g, 1171.5±34.0g, 1228.8±57.2g, 1250.5±27.9g and 964.0±23.5g, respectively. The highest body weight within the Groups A, B, C and D on day 21pi were A2 (1116.3±58.1g), B3 (1110.5±21.2g), C1 (1411.78±43.8g) and D3 (1250.5±27.9g), respectively. Overall, there was no significant differences ($p>0.05$) between groups on day 7 pi and some significant different ($p<0.05$) between groups on day 21 pi. On day 21 pi, there was no significant differences ($p>0.05$) within group. However, there were significant different ($p<0.05$) between group C1 to group A1, A2, A3, B1, B2, B3, D1 and E. Group B1 was also different ($p<0.05$) with group C1, C2, C3, D2 and D3. Group C2 was different with group B1, B2 and E. Group E was different with C1, C2, C3, D1, D2 and D3. (Figure 2 and Appendix 2).

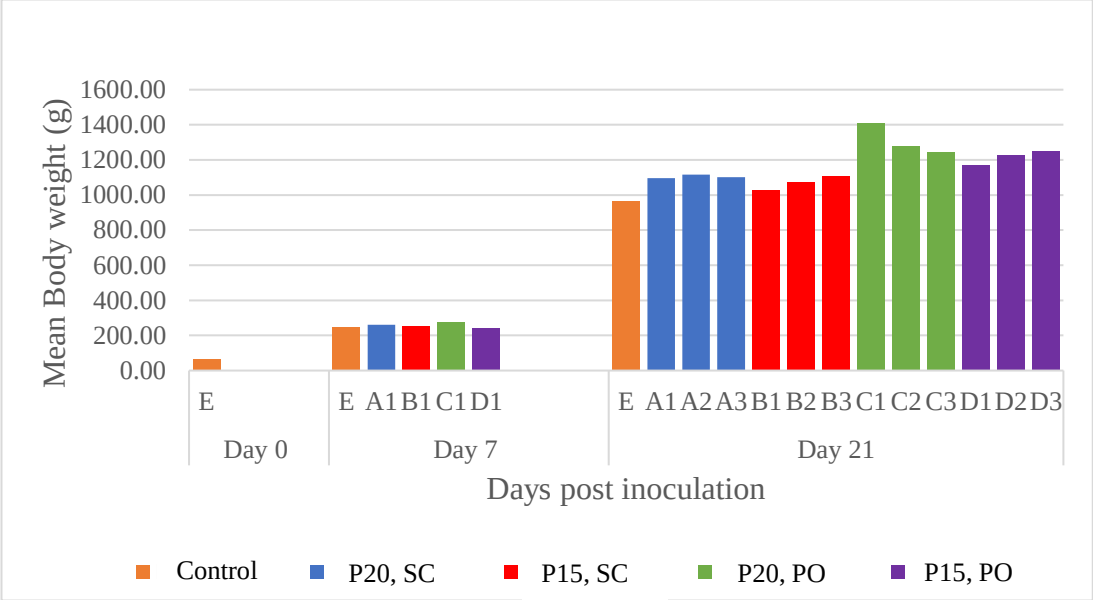


Figure 2: Body weight of chickens throughout the trial.

4.3 Liver Weight

Liver weight of the chickens in all the groups increased significantly from day 0 to day 21 pi. The liver weight of DOC was 2.9±0.1g, then increased to 10.0±0.7g on day 7 pi and 19.8±1.8g on day 21 pi for Group E. On day 7 pi, the liver weight of the chickens in Groups A1, B1, C1, D1 and E were 9.5±0.7g, 10.0±0.7g, 9.5±0.5g, 9.0±9.8g and 10.0±0.7g respectively. Group B1 had the highest liver weight (10.0±0.7g) on day 7 pi. On day 21 pi, the liver weight of the chickens in Groups A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3 and E were 23.0±1.7g, 19.5±1.2g, 21.3±1.5g, 18.8±1.0g, 19.3±0.3g, 20.3±1.3g, 25.8±1.7g, 22.3±1.0g, 22.5±0.7g, 23.5±0.7g, 20.0±0.4g, 20.0±1.4g and 19.8±1.8g, respectively. The highest liver weight within the group A, B, C and D on day 21pi were A1 (23.0±1.7g), B3 (20.3±1.3g), C1 (25.8±1.7g) and D1 (23.5±0.7g) respectively. Overall, there was no significant different ($p>0.05$) between groups on days 7, within and between groups on day 21 pi. (Figure 3 and Appendix 3).

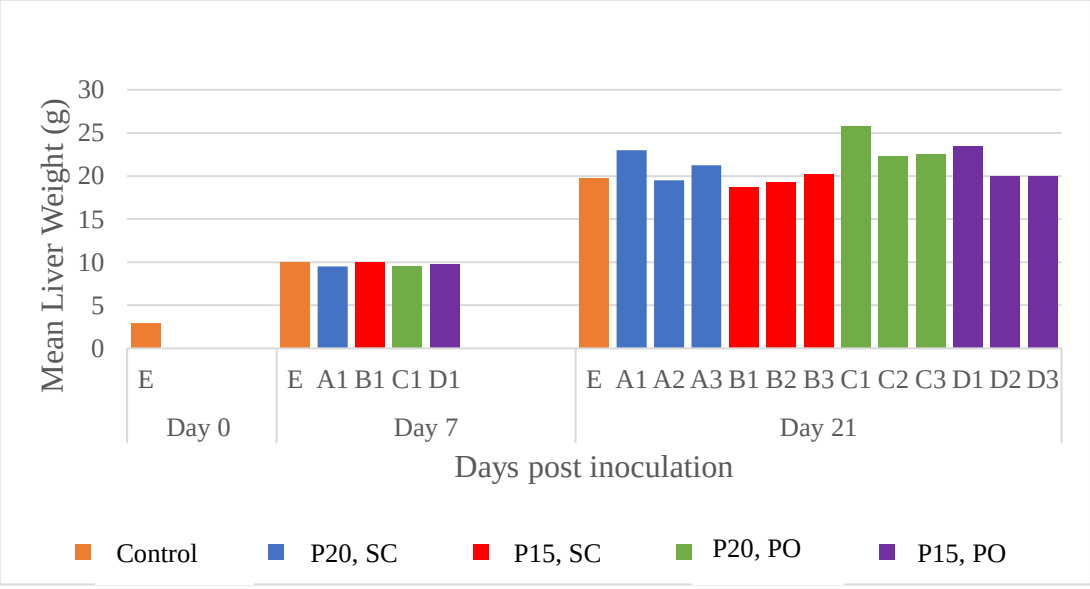


Figure 3: Liver weight of chickens throughout the trial.

4.4 Liver to Body Weight Ratio (10^{-2})

The liver to body weight ratio of the DOC was 4.29±0.16. On day 7 pi, the liver to body weight ratio of the chickens in Groups A1, B1, C1, D1 and E were 3.63±0.07, 4.02±0.25, 3.40±0.09, 4.00±0.23 and 4.00±0.23, respectively. Group B1 had the highest liver to body weight ratio (4.02±0.25) on day 7 pi. On day 21 pi, the liver to body weight ratio of chickens in Groups A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3 and E were 2.09±0.11, 1.75±0.07, 1.92±0.09, 1.82±0.10, 1.80±0.05, 1.82±0.09, 1.83±0.13, 1.75±0.11, 1.81±0.03, 2.01±0.07, 1.63±0.05, 1.59± 0.08 and 2.04±0.13, respectively. The highest liver to body weight ratio within the group A, B, C and D on day 21pi were A1 (2.09±0.11), B1 (1.82±0.1), B3 (1.82±0.09), C1 (1.83±0.13) and D1 (2.01±0.07) respectively. Overall, there was no significant different ($p>0.05$) between groups on days 7, within and between groups on day 21 pi. (Figure 4 and Appendix 4).

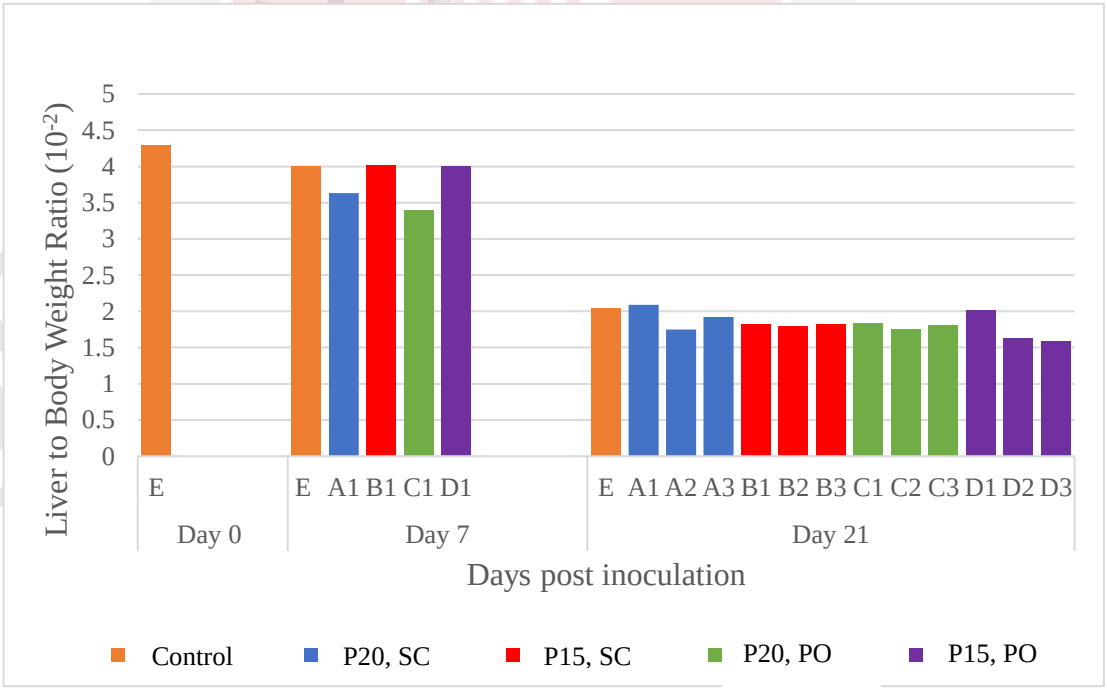


Figure 4: Liver to body weight ratio (10^{-2}) of chickens throughout the trial.

4.5 Gross lesions

4.5.1 Day 0 pi

The livers of day old chick (DOC) from Group E (Control) appeared yellowish (Figure 5).

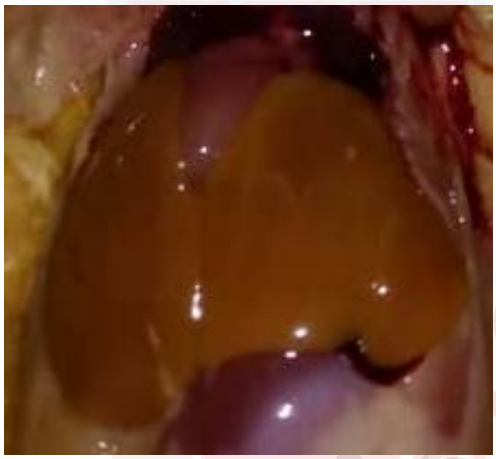


Figure 5: Normal liver of chicks on day 0 pi in Group E (Control).

4.5.2 Day 7 pi

The liver of chicks from Groups A1, B1, C1, D1 and E appeared dark red, glistening with sharp edges, indicating no enlargement or inflammation of the livers. No significant gross lesions were observed in all groups of chicks. (Figure 6).

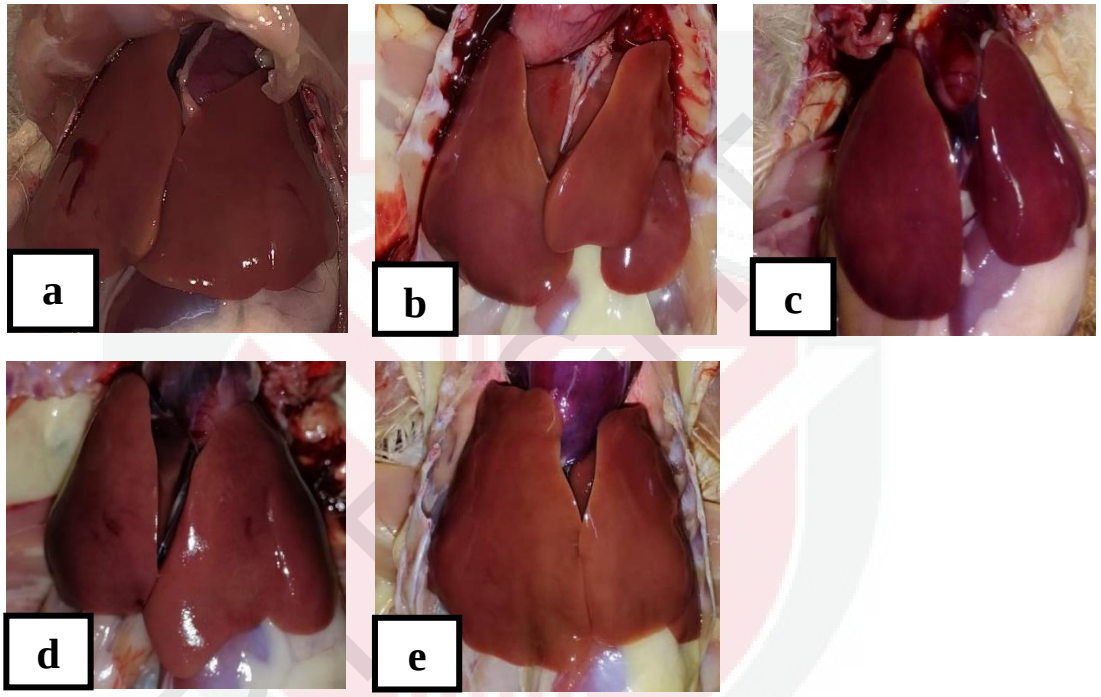


Figure 6: Normal livers of chicks on day 7 pi. (a). Group A1, (b). Group B1, (c). Group C1, (d). Group D1 and (e). Group E.

4.5.3 Day 21 pi

The liver of chickens from Groups A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3 and E appeared dark red, glistening with sharp edges, indicating no enlargement or inflammation of the livers. No significant gross lesions were observed in all groups of chicks (Figure 7 and 8).

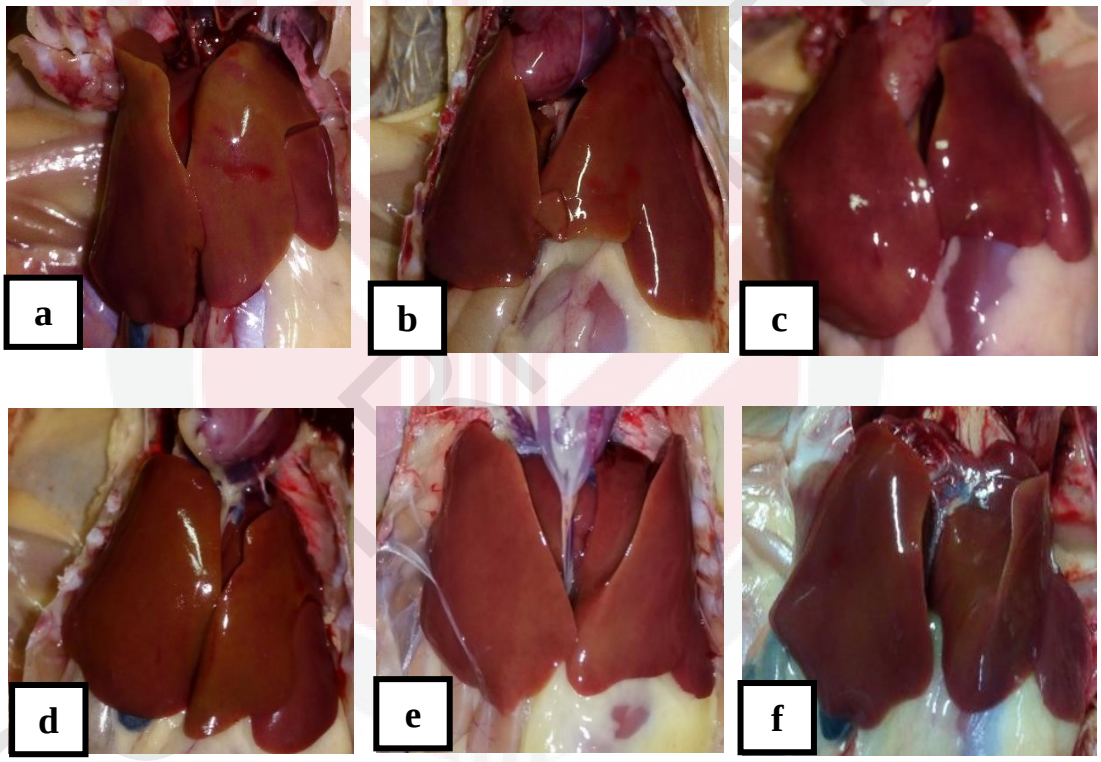


Figure 7: Normal livers of chickens on day 21 pi. (a). Group A1, (b). Group A2, (c). Group A3, (d). Group B1 and (e). Group B2 and (f). Group B3.

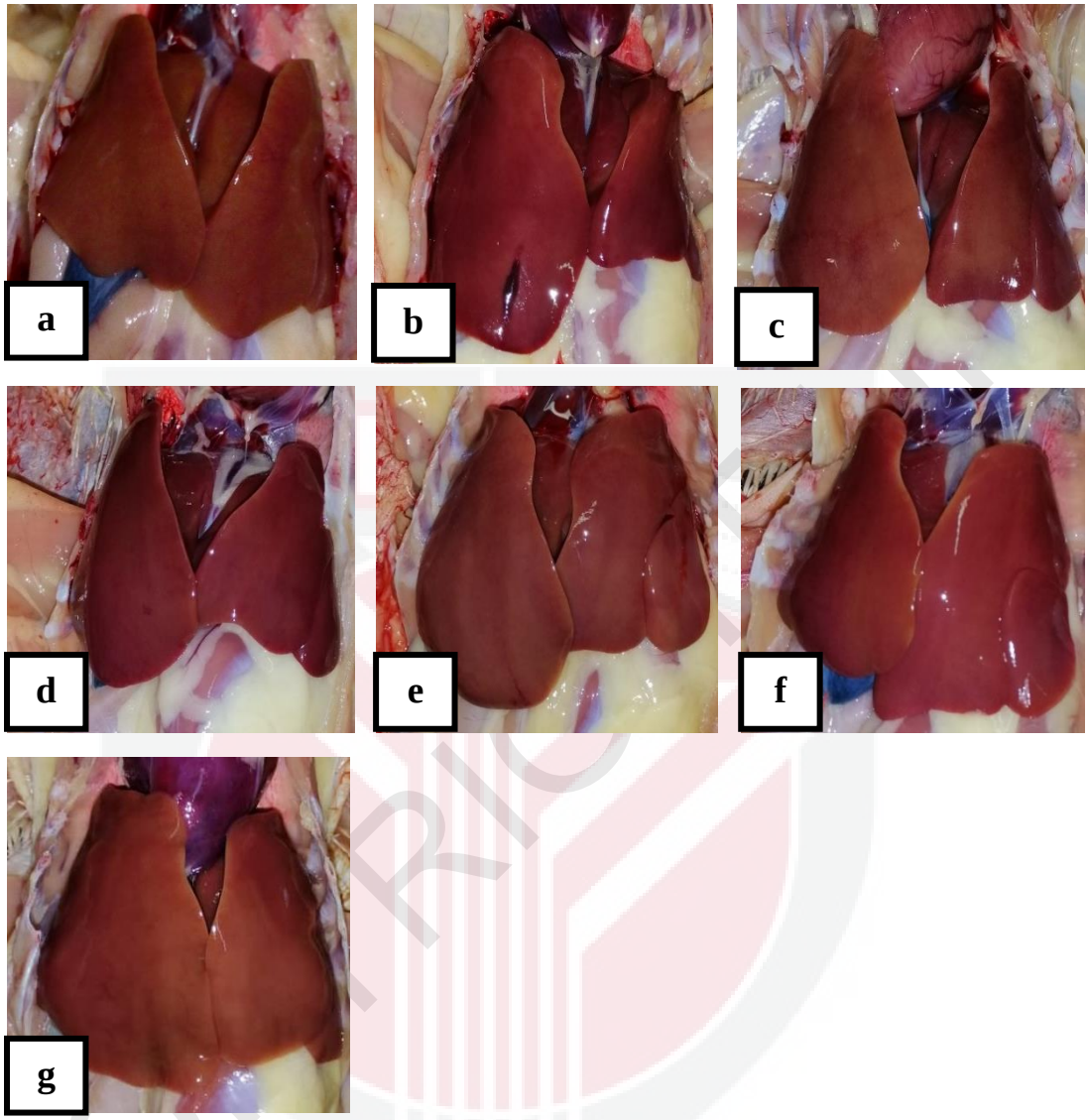


Figure 8: Normal livers of chickens on day 21 pi. (a). Group C1, (b). Group C2, (c). Group C3, (d). Group D1 and (e). Group D2, (f). Group D3 and (g). Group E.

4.6 Histological Lesions

4.6.1 Day 0 pi

Multiple vacuoles were observed on the histology of liver of DOC from Group E (Control). No intranuclear inclusion bodies (INIB) were found in the hepatocytes. No significant histological lesion was observed (Figure 9).

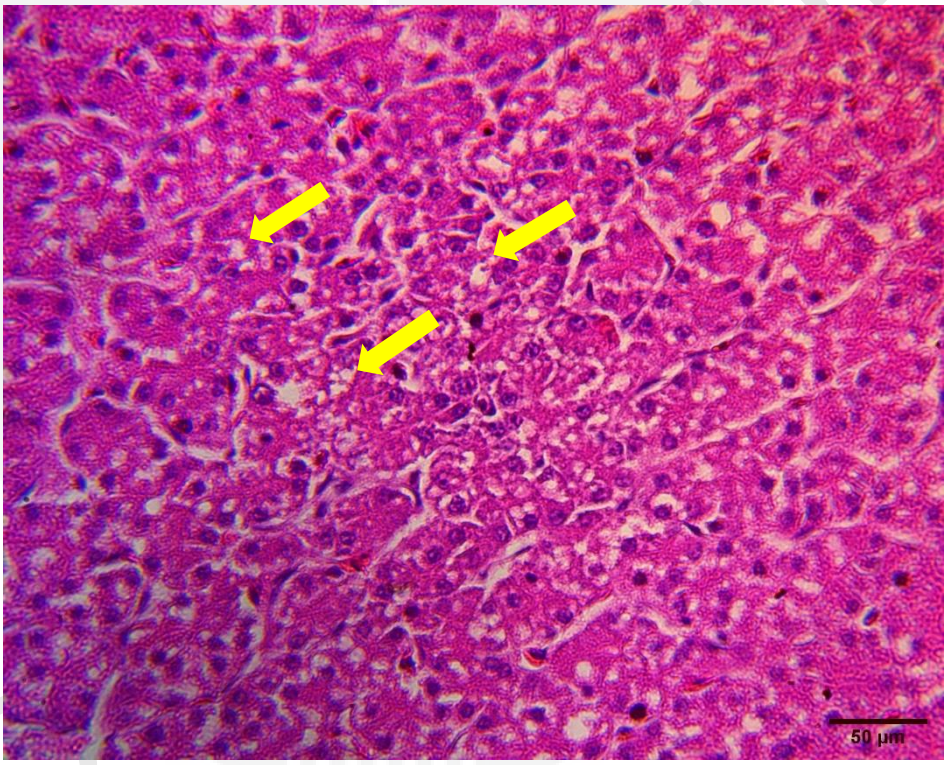


Figure 9: Normal histology of liver on day 0 pi in Group E (Control). Multiple vacuoles present within the hepatocytes (yellow arrows). HE, 40×. Bar= 50µm.

4.6.2 Day 7 pi

No intranuclear inclusion bodies (INIB) were present in the hepatocytes. No histological lesions were observed in all groups of chicks (Figure 10).

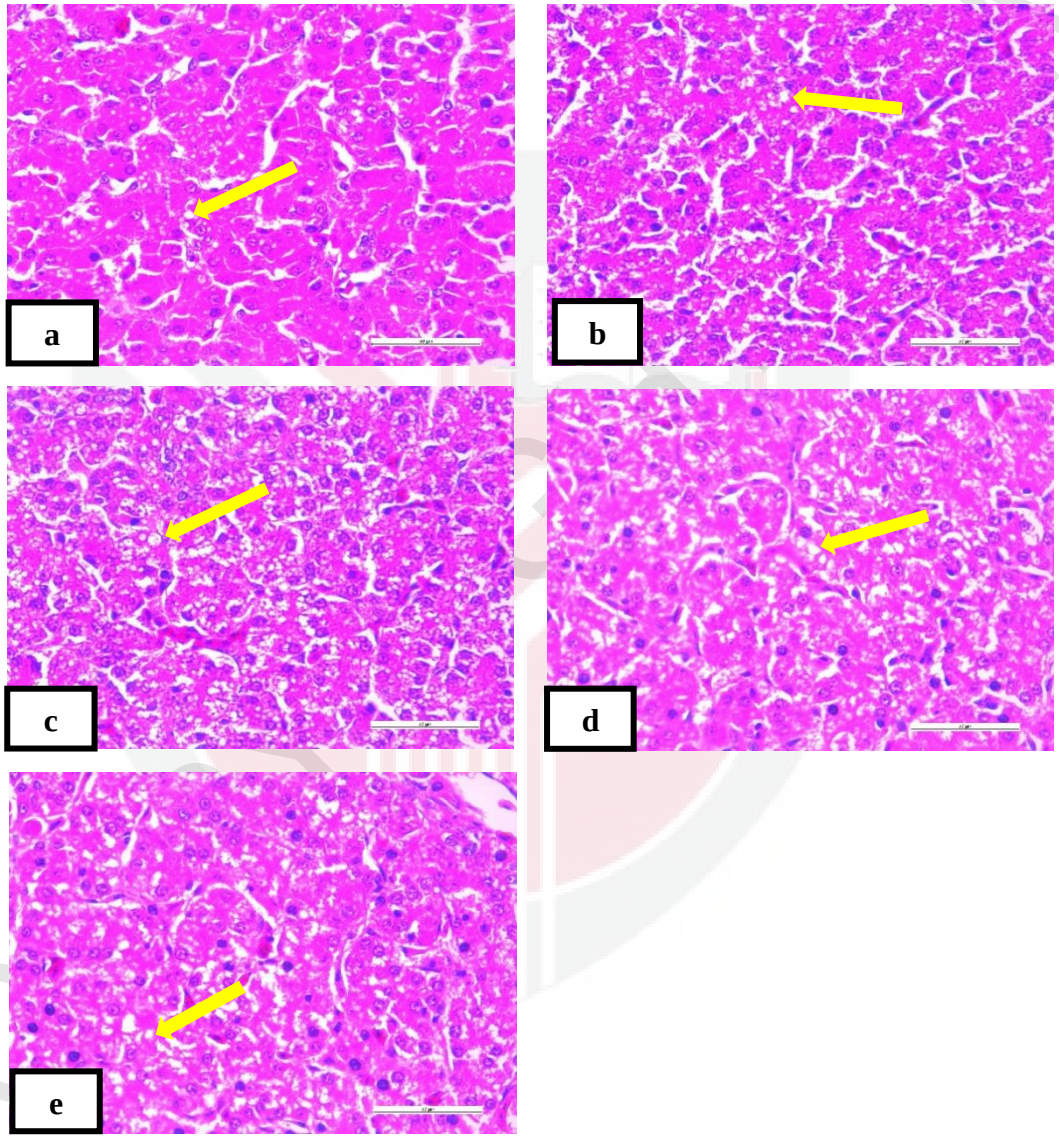


Figure 10: Normal histology of liver of chickens on day 7 pi. Fever vacuoles present in the organs (yellow arrows). (a). Group A1, (b). Group B1, (c). Group C1, (d). Group D1 and (e). Group E. HE, 40×. Bar = 50µm.

4.6.3 Day 21 pi

No intranuclear inclusion bodies (INIB) were present in the hepatocytes. No histological lesions were observed in all groups of chicks (Figure 11 and 12).

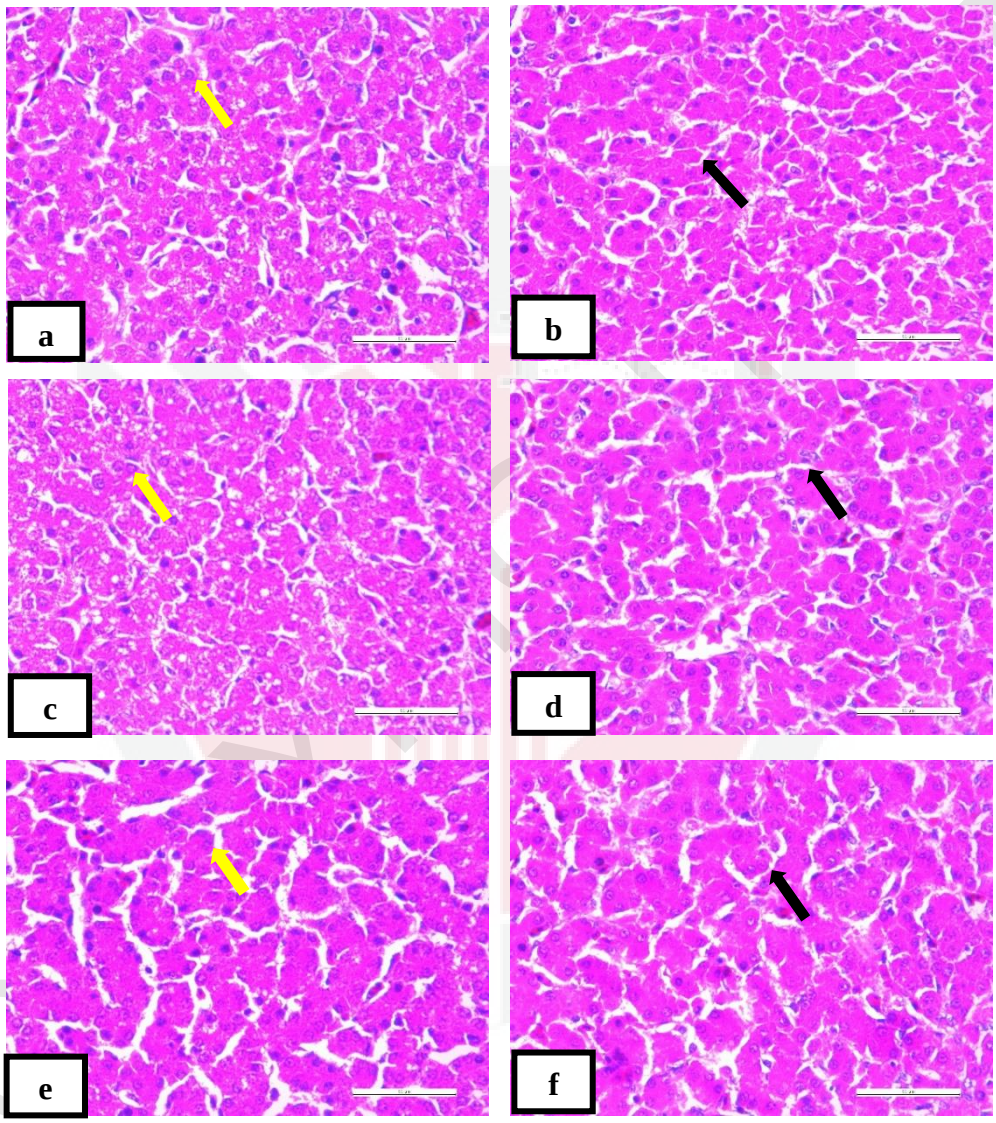


Figure 11: Normal histology of liver of chickens on day 21 pi. Presence of hepatocytes (yellow arrows) and sinusoids (black arrows) in the organs. (a). Group A1, (b). Group A2, (c). Group A3, (d). Group B1, (e). Group B2 and (f). Group B3. HE, 40×. Bar = 50µm.

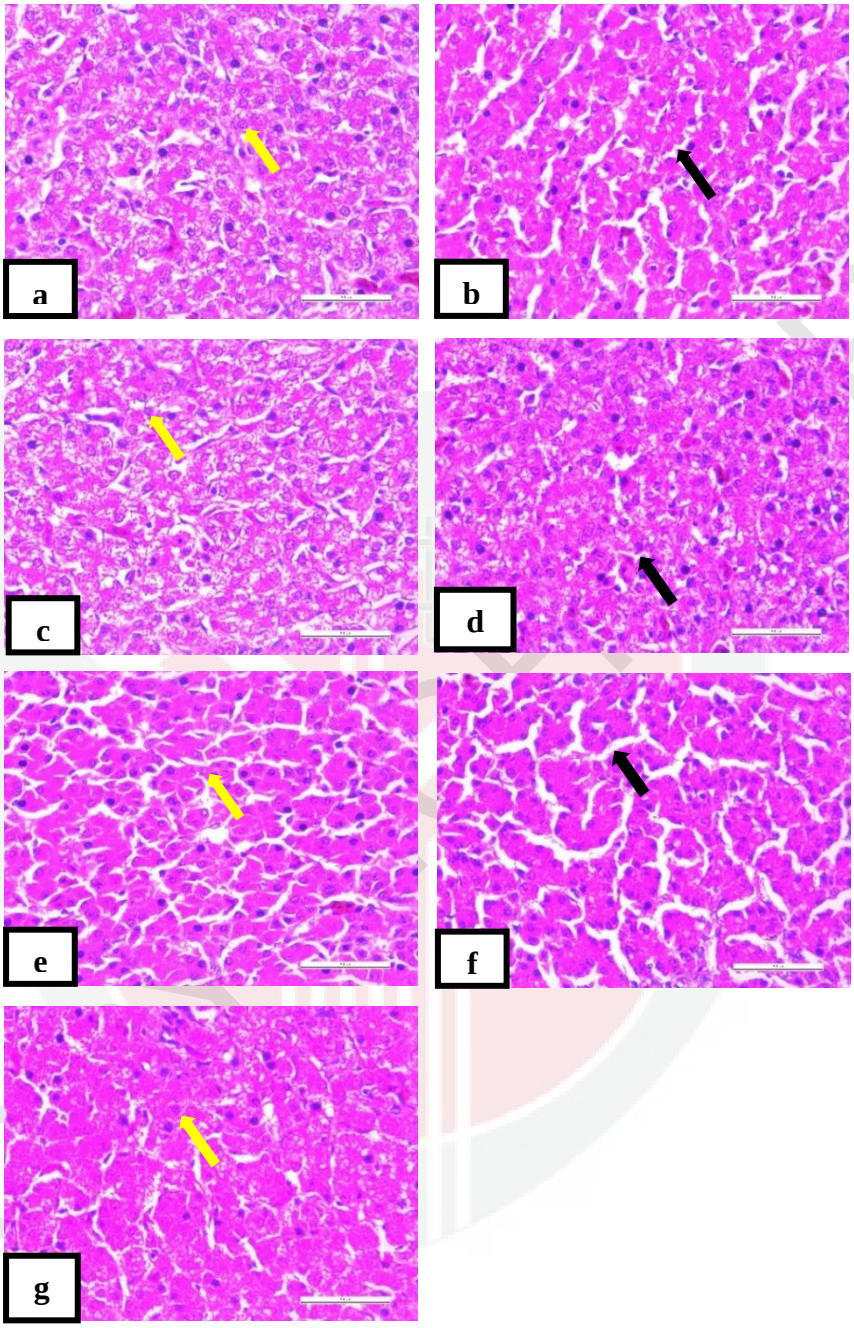


Figure 12: Normal histology of liver of chickens on day 21 pi. Presence of hepatocytes (yellow arrows) and sinusoids (black arrows) and in the organs. (a). Group A1, (b). Group A2, (c). Group A3, (d). Group B1, (e). Group B2, (f). Group B3, and (g). Group E. HE, 40×. Bar = 50µm.

4.7 Fowl Adenovirus Antibody Titer

The FAdV antibody titer was 5672±1275 in Group E (Control) on day 0 pi and decreased to 1256±730 on day 7 pi and 236±88 on day 21 pi. On day 7 pi, Group C1 showed the highest titer at 1557±528. However, it dropped to 347±91 on day 21 pi. Similar decreased of antibody titer value from day 7 pi to day 21 pi was observed in Group B1 (from 771±289 to 567±277) and D1 (895±506 to 231±94). For Group A1, the antibody titer increased from 380±99 to 486±362 from day 7 pi to day 21 pi. On day 21 pi, the highest antibody within each Group A, B, C and D were A3 (969±420), B2 (1956±916), C2 (1003±566) and D2 (958±492), respectively. There is no significant difference ($p>0.05$) among and within all groups of chickens throughout the 21 days of trial (Figure 13 and Appendix 5).

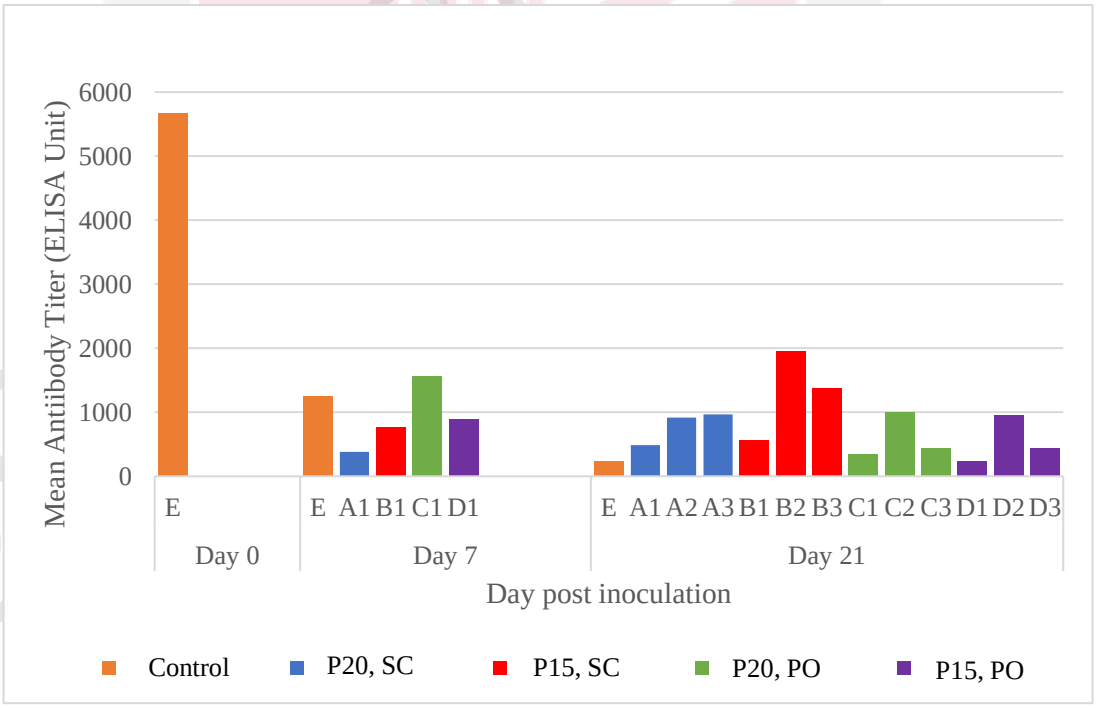


Figure 13: Fowl adenovirus antibody titer of chickens throughout the trial.

4.7.1 Fowl Adenovirus Antibody Titer between subgroup on Day 7 pi and Day 21 pi

On day 7 pi, Group C1 had the highest antibody titer (1557±528) among others subgroup. On day 21 pi, Group B1 had the highest antibody titer (567±277) among Group A1, B1, C1 and D1; Group B2 had the highest antibody titer (1956±916) among Group A2, B2, C2 and D2; Group B3 had the highest antibody titer (1383±541) among Group A3, B3, C3 and D3. There is no significant difference ($p>0.05$) between all groups of chickens on both day 7 pi and day 21 pi. (Figure 14 and Appendix 5).

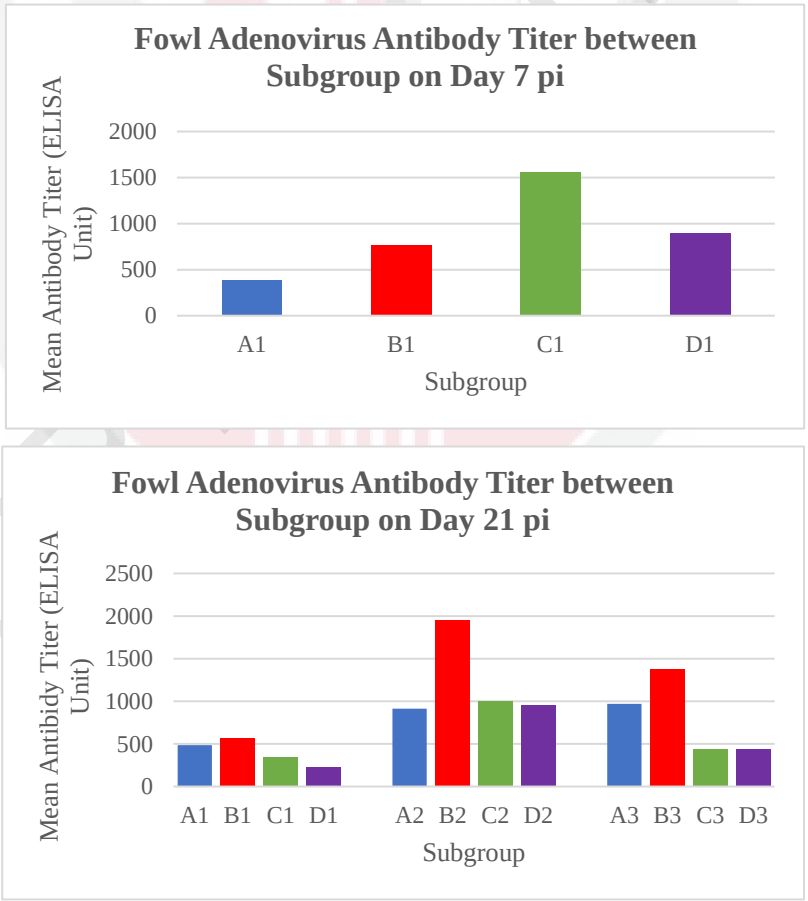


Figure 14: Fowl adenovirus antibody titer of chickens between subgroup on day 7 pi and day 21 pi.

4.7.2 Fowl Adenovirus Antibody Titer within Group

For Group A, the highest antibody titer within all subgroup from both day 7 pi and day 21 pi was A3 (969±420). For Group B, the highest antibody titer within all subgroup from both day 7 pi and day 21 pi was B2 (1956±916). For Group C, the highest antibody titer within all subgroup from both day 7 pi and day 21 pi was C1 (1557±528) from day 7 pi. For Group D, the highest antibody titer within all subgroup from both day 7 pi and day 21 pi was D2 (958±492). There is no significant difference ($p>0.05$) within all groups of chickens throughout the 21 days of trial (Figure 15 and Appendix 5).

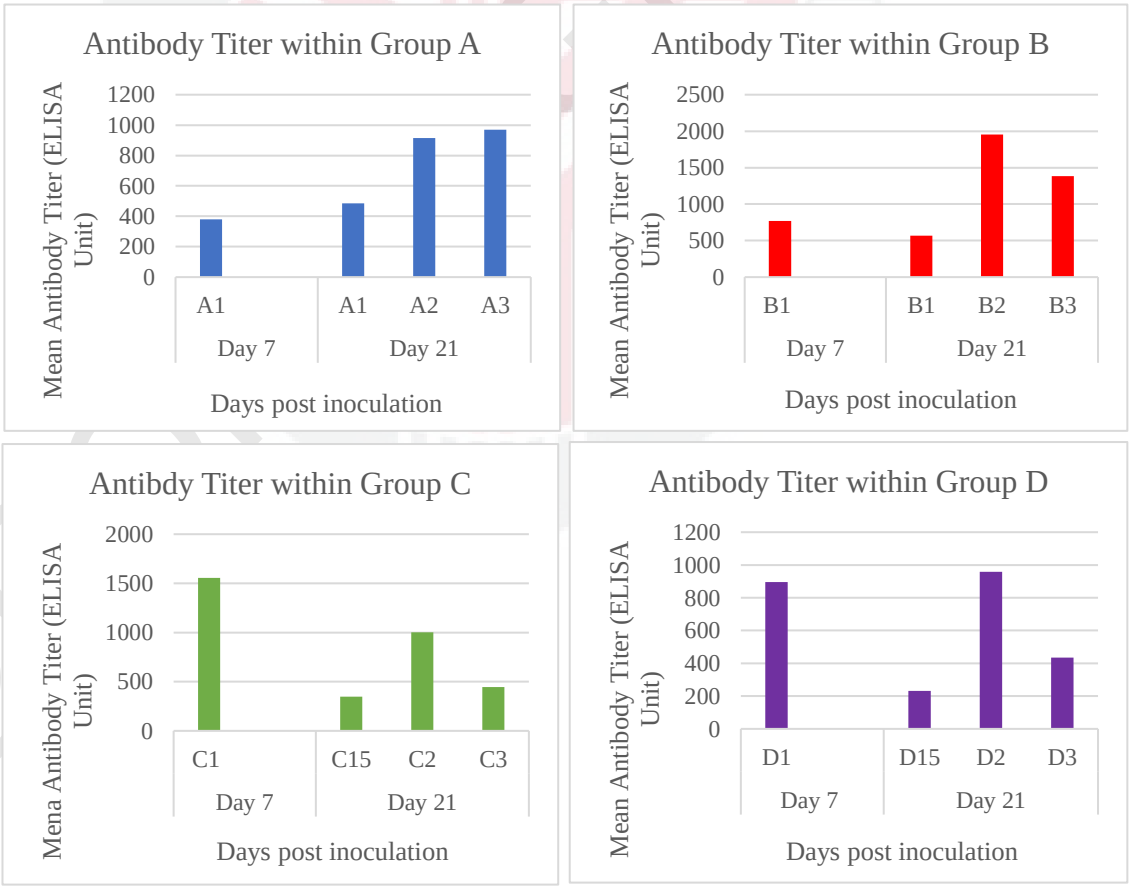


Figure 15: Fowl adenovirus antibody titer within each group.

5.0 DISCUSSION

The safety of attenuated FAdV in this study is evaluated by five parameters, which were the clinical signs, body weight, liver weight, liver to body weight ratio, gross lesions and histological lesions. Attenuated FAdV contain a version of living pathogenic microbe that has been attenuated or weakened in the lab through serial passaging in a foreign host. The extended passaging introduces some mutations which the mutated pathogen is significantly different from the pathogenic form, it will not cause disease in the original host but can induce the immune response effectively. Hence, when a FAdV isolate is not fully attenuated, it can lead to FAdV infection in chickens.

All the chickens were healthy and active eating and drinking without showing any abnormal clinical signs caused by FAdV infection such as ruffled feathers, crouching, lethargy and sudden death throughout the 14 days trial. The body weight of all chickens increases significantly across the days, consistently with the liver weight. This support by the theory of the heavier the chickens, the heavier liver is. According to Mark et al. (2007), FAdV is known to cause Inclusion body hepatitis (IBH) which will cause reduced in feed conversion and then lead to poor growth, weight gain and eventually cause mortality. Body weight of group E (control) was statistically significant ($p<0.05$) with group C1, C2, C3, D1, D2 and D3. Group E (control) also recorded the lowest body weight among all the group on day 21 pi. There were no significant differences ($p>0.05$) of liver weight of all the groups on both day 7 and day 21 pi. Hence, all attenuated FAdV with P15 and P20 inoculated subcutaneously and orally were safe to use as it did not cause any effect in term of body weight and liver

weight of chickens. In addition, the chickens in all groups showed normal growth rate and systemic weight gain, indicating attenuated FAdV is not interfering with the growth performance of the chickens.

Furthermore, Steer et al. (2011) suggested that liver to body weight ratio is a good supportive assessment of the protective efficacy of an attenuated FAdV-8b and FAdV-11 vaccine. This parameter can detect any enlargement of the liver due to viral replication in the organ more accurately compared to measuring liver weight alone. According to Adair and Fitzgerald (2008), the FAdV main replicate in the liver. During the growth phase, the ratio of liver weight to body weight reach a peak at about one to two weeks of age and then decreases gradually with age until 8 weeks as the liver started developing into maximum size.

Besides, upon necropsy examination, all the livers of chickens were in dark red, appear moist, glistening with sharp edges, indicating no enlargement and inflammation. Thus, there were no characteristics of intranuclear inclusion bodies of FAdV found in all liver samples during histological examination except from the sample from the day-old chicks. The histological result of day-old chicks shown fatty liver which characterized by the presence of vacuolation caused by presence of cholesterol. Hence, it was suggested that attenuated FAdV was safe to be used where it not caused any gross and histological in chickens.

Moreover, the immunogenicity is evaluated based on the antibody titer induced by attenuated FAdV in commercial broiler chickens. On day 0 pi, the control groups revealed a high antibody titer that can caused by presence of maternal-derived antibody (MDA) from the breeder flock. As we know, MDA will not last longer, thus

the antibody titer of the control group dropped at the following weeks from 5672±1275 (day 0 pi) to 1256±730 (day 7 pi) and eventually become 236±88 (day 21 pi). Almost all groups (group B1, C1 and D1) that vaccinated on day 0 had a higher antibody titer on day 7 pi (B1- 771±289; C1- 1557±528; D1- 895±506) than day 21 pi (B1- 567±277; C1- 347±91; D1- 231±94), which shown drop of antibody titer across the day, indicating the MDA interfered with the immune response produced by vaccine. On day 21 pi, almost all groups with booster (group B2, C2 and D2) had the highest antibody titer within the group, which were 1956±916, 1003±566 and 958±492, respectively, indicating booster dose can induce a higher immune response. On day 21 pi, the group B2 which was inoculate with P15 attenuated FAdV vaccine subcutaneously on day 0 pi and given a booster dose on day 7 pi had the highest antibody titer (1956±916) among all the other groups. Sohaimi et al. (2019) reported that FAdV with lower passage has higher virus titer than higher passage. There were no significant different between route of inoculation either via oral or subcutaneous route in term of induction of the antibody titre (Sohaimi et al., 2018). However, there was no significantly differences of antibody titer ($p>0.05$) neither within nor between group across the different time.

6.0 CONCLUSION

The study suggested that attenuated FAdV at passage 15 and inoculated subcutaneously on day old and given a booster dose on day 7 post inoculation was able to induce the highest antibody titer compared to all the other groups. The attenuated FAdV with P15 and P20 inoculated subcutaneously and orally were safe to use without causing any side effects to the commercial broiler chickens.

7.0 RECOMMENDATIONS

For further study to be done, the number of sample animals should be increase to obtain a more uniform and standard results. Specific pathogen free (SPF) chickens were highly recommended to avoid interference of maternal-derived antibodies or history of previous exposure to the virus. Lastly, efficacy study should conduct by challenging the vaccinated chickens with the virus to determine the effectiveness of the vaccine.

REFERENCES

Adair, B.M., and S.D. Fitzgerald (2008). Group I Adenovirus Infections. In: *Diseases of Poultry*, 12th ed. Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougals, L.R., Nolan, L.K. and Swayne, D.E. (eds). Wiley-Blackwell, Hoboken, NJ. pp 252-262.

Brown Jordan, A., Gongora, V., Hartley, D. and C. Oura (2018). A Review of Eight High-Priority, Economically Important Viral Pathogens of Poultry within the Caribbean Region. Veterinary sciences, 5(1), 14. https://doi.org/10.3390/vetsci5010014

Charania, N.A. and S.M. Moghadas (2017). Modelling the effects of booster dose vaccination schedules and recommendations for public health immunization programs: the case of Haemophilus influenzae serotype b. *BMC Public Health*, 17(1):705. https://doi.org/10.1186/s12889-017-4714-9.

Chen, L., Yin, L., Zhou, Q., Peng, P., Du, Y., Liu, L., Zhang, Y., Xue, C. and Y. Cao (2019). Epidemiological investigation of fowl adenovirus infections in poultry in China during 2015–2018. *BMC Vet Res*, 15: 271 https://doi.org/10.1186/s12917-019-1969-7.

Cizmecigil, U. Y., Umar, S., Yilmaz, A., Bayraktar, E., Turan, N., Tali, B., Aydin, O., Tali, H. E., Yaramanoglu, M., Yilmaz, S. G., Kolukisa, A., Sadeyen, J. R., Iqbal, M., and H. Yilmaz (2020). Characterisation of fowl adenovirus (FAdV-8b) strain concerning the geographic analysis and pathological lesions associated with inclusion body hepatitis in broiler flocks in Turkey. *Journal of Veterinary Research*, 64(2), 231–237. https://doi.org/10.2478/jvetres-2020-0026.

Cowen, B. (1992). Inclusion body hepatitis-anemia and hydropericardium syndromes: aetiology and control. *World's Poultry Science Journal*, 48: 247-254.

Dawson G.J., Orsi L.N., Yates V.J., Chang P.W. and A.D. Pronovost (1980). An enzyme-linked immunosorbent assay for detection of antibodies to avian adenovirus and avian adenovirus-associated virus in chickens. *Avian. Dis*. 24: 393-402

Ekanayake, S. (2009). Inclusion body hepatitis as a primary disease in commercial broiler chickens (Master's thesis, University of Saskatchewan, Saskatoon). Retrieved from https://core.ac.uk/download/pdf/226115185.pdf

Fischer A.H., Jacobson K.A., Rose J. and R. Zeller (2008). Paraffin Embedding Tissue Samples for Sectioning. *CSH Protocols*, http://cshprotocols.cshlp.org/content/2008/5/pdb.prot4989.

Gupta, A. (2018). Pathogenesis and control of inclusion body hepatitis in broiler chickens (Doctoral dissertation, University of Saskatchewan, Saskatoon). Retrieved from https://harvest.usask.ca/bitstream/handle/10388/8562/GUPTA-DISSERTATION-2018.pdf?sequence=1&isAllowed=y

Hafez, H.M. (2011). Avian adenoviruses infections with special attention to inclusion body hepatitis/hydropericardium syndrome and egg drop syndrome. *Pakistan Veterinary Journal*, 31(2): 85-92.

Hair-Bejo, M. (2005). Inclusion body hepatitis in a flock of commercial broiler chickens. *Journal of Veterinary Malaysia*, 17:23-26.

Helena, G., Cynthia, P., Davor, O., and N. Eva (2006). Study of vertical transmission of Fowl Adenoviruses. *Can Vet Res*, 70(3): 230-233.

Helmboldt, C.F. and M.N. Frazier, (1963). Avian hepatic inclusion bodies of unknown significance. *Avian Diseases*, 7:446-450.

Hess, M. (2019). Aviadenovirus infections. In: *Diseases of Poultry*, 14th ed. Swayne, D.E., Boulianne, M., Logue, C.M., McDougald, L.R., Nair, V. and Suarez, D.L. (eds). Wiley-Blackwell, pp 322-332.

Hess, M. (2013). Aviadenovirus infections. In: *Diseases of Poultry*, 13th ed. Swayne, D.E., Glisson, J.R., McDougald, L.R., Nolan, L.K., Suarez, D.L., and V.L. Nair (eds). Wiley-Blackwell.

Hess, M., Raue, R., and H.M. Hafez (1999). PCR for specific detection of haemorrhagic enteritis of turkeys, an avian adenovirus. *J Virol Meth*. 81: 199-203.

Lauring, A.S., Jones, J.O. and R. Andino, (2010). Rationalizing the development of live attenuated virus vaccines. Nature Biotechnology, 28(6): 573–579. https://doi.org/10.1038/nbt.1635.

Li, P.H., Zheng P.P, Zhang, T.F., Wen, G.Y., Shao, H.B., and Q.P. Luo (2017). Fowl adenovirus serotype 4: Epidemiology, pathogenesis, diagnostics detection, and vaccine strategies. *Poultry Science*, 96(8): 2630-2640.

Mark, P., Paul, F.M., Janet, M.B. and J.A. Dennis (2007), 6th ed. *Poultry Diseases*, pp367-381.

McFerran, J.B. and Adair B.McC. (1977) Avian adenoviruses - a review, *Avian Pathology*, 6:3, 189-217. https://doi.org/10.1080/03079457708418228

McFerran, J.B. and Smyth J.A. (2000). Avian adenovirus. *Rev., Sci. Tech. Off. Int. Epiz*., 19(2):589-601.

Niczyporuk, J.S. (2018). Adenoviruses and Their Diversity in Poultry. In: *Application of Genetics and Genomics in Poultry Science*. Xiaojun.L (eds).

Norina, L., Norsharina, A., Nurnadiah, A.H., Redzuan, I., Ardy, A., and I. Nor-ismaliza, (2006), Avian adenovirus isolated from broiler affected with inclusion body hepatitis. *Journal of Veterinary Malaysia*, 7, 121-126.

Saifuddin, M. and C.R., Wilks (1992). Effects of fowl adenovirus infection on the immune system of chickens. *J. Comp. Pathol*. 107:285–294.

Schonewile, E., Singh, A., Gobel, T.W., Gerner, W., Saalmuller, A., and M. Hess (2008). Fowl adenovirus (FadV) serotype 4 causes depletion of B and T cells lymphoid organs in specific-pathogen-free chickens following experimental infection. *Veterinary Immunology and Immunopathology*, 121(1-2): 130-139. doi: 10.1016/j.vetimm.2007.09.017.

Scott, D.F. (2017). Adenovirus infections. *Diseases of Poultry*, 281-331.

Sohaimi, N.M., Bejo, M.H., Omar, A.R., Ideris,A., and N.M. Isa (2018). Hexon and fiber gene changes in an attenuated fowl adenovirus isolate from Malaysia in embryonated chicken eggs and its infectivity in chickens. *Journal of Veterinary Science*, 19(6): 759–770. https://doi.org/10.4142/jvs.2018.19.6.759.

Sohaimi, N.M., Bejo, M.H., Omar, A.R., Ideris, A. and N.M. Isa (2019). Molecular characterization of fowl adenovirus isolate of Malaysia attenuated in chicken embryo liver cells and its pathogenicity and immunogenicity in chickens. *PLoS ONE*, 14(12): e0225863. https://doi.org/10.1371/journal.pone.0225863.

Steer, P.A., D.O'Rourke, S.A. Ghorashi, and A.H. Noormohammadi (2011). Application of high-resolution melting curve analysis for typing of fowl adenoviruses in field cases of inclusion body hepatitis. *Aust Vet J*. 89(5): 184-192.

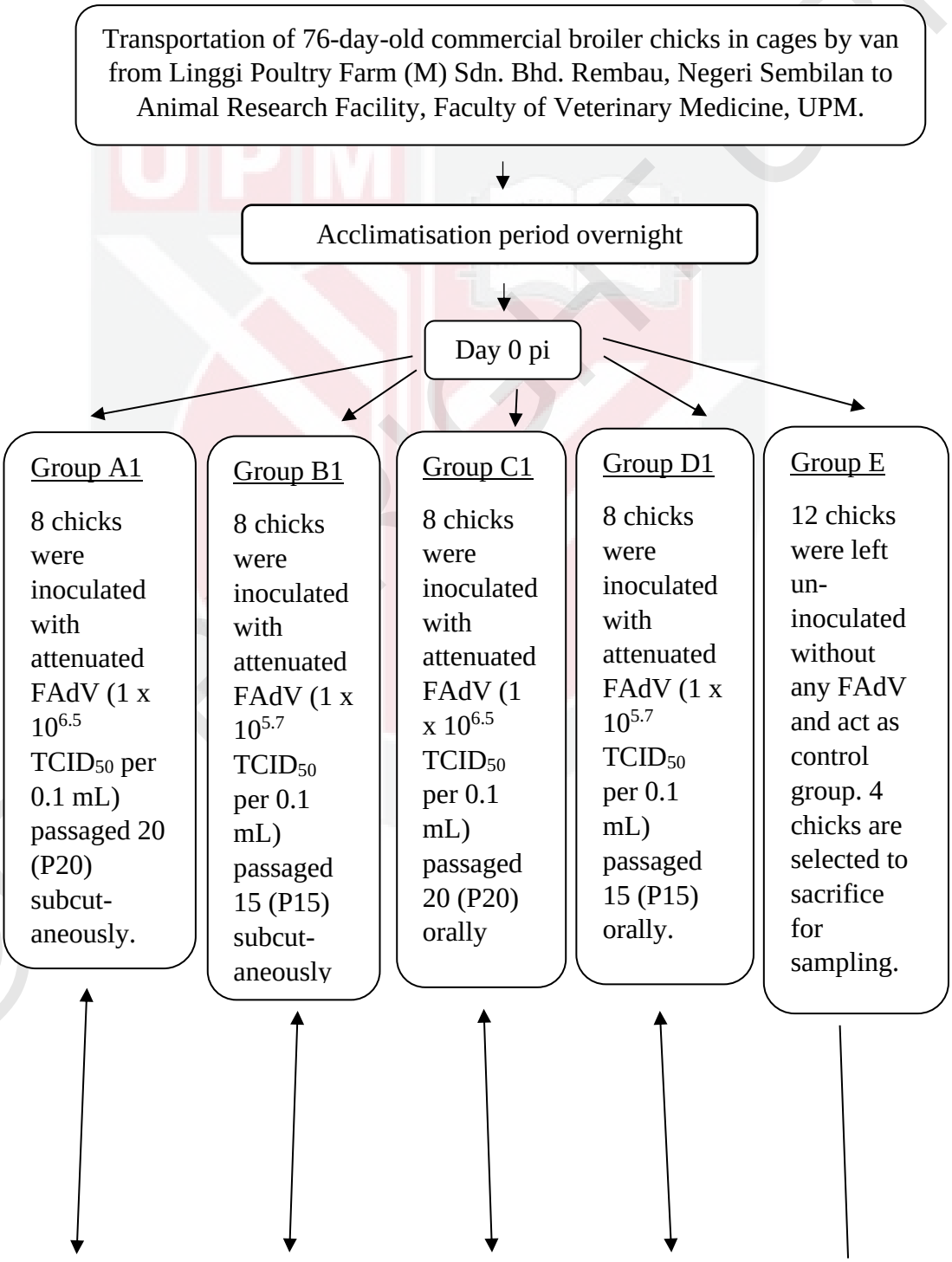
Steer, P.A., Kirkpatrick, N.C., O'Rourke, D., and A.H. Noormohammadi (2009). Classification of Fowl adenovirus serotypes by use of high-resolution melting curve analysis of the hexon gene region. *Journal of Clinical Microbiology*. 47(2) 311-321.

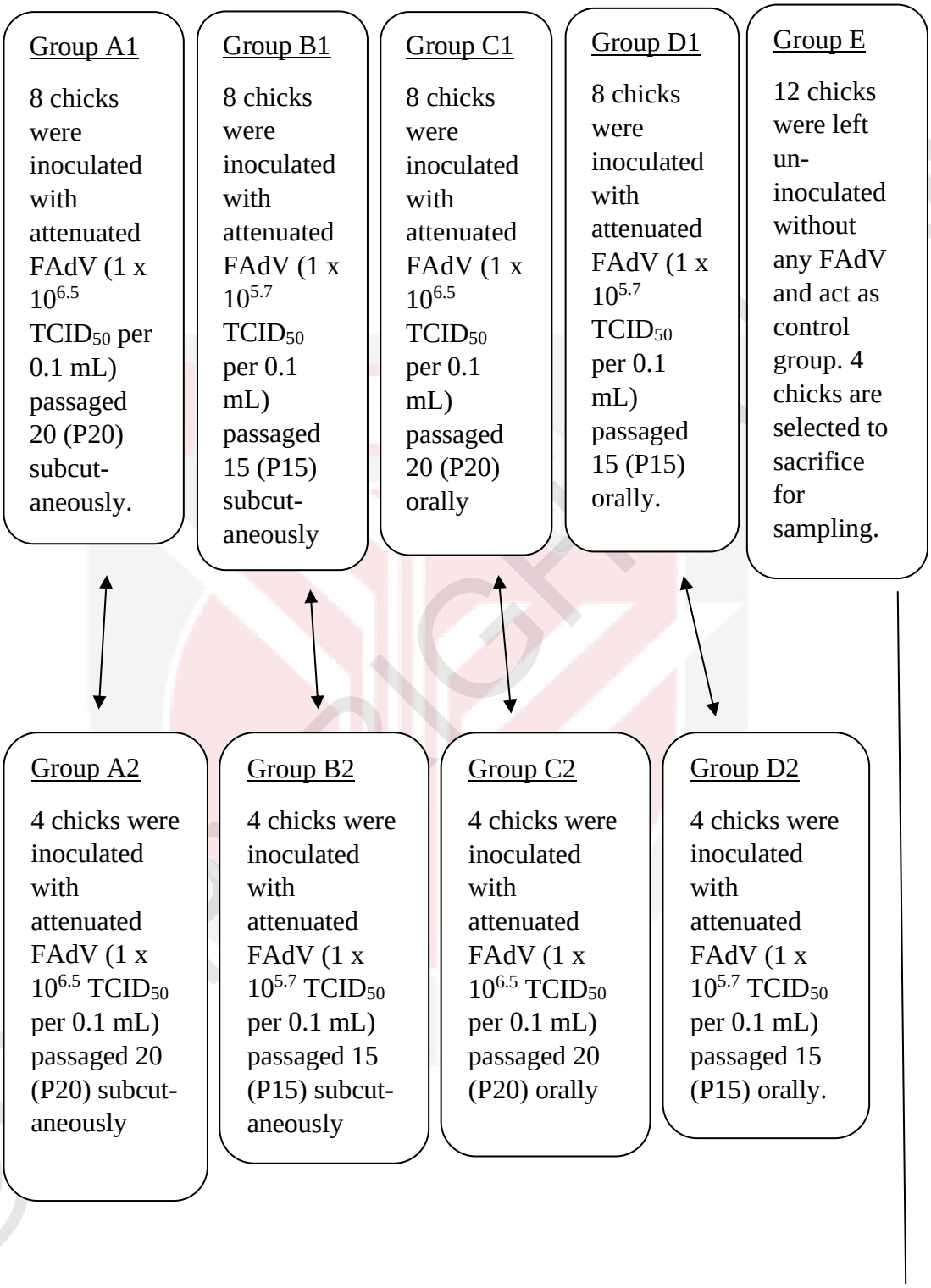
Toro, H., Gonzalez, C., Cerda, L., Hess, M., Reyes, E., and C. Geisse (2000). Chicken anemia virus and Fowl adenoviruses: association to induce the Inclusion body hepatitis or Hydropericardium syndrome. *Avian Diseases*, 44(1): 51.

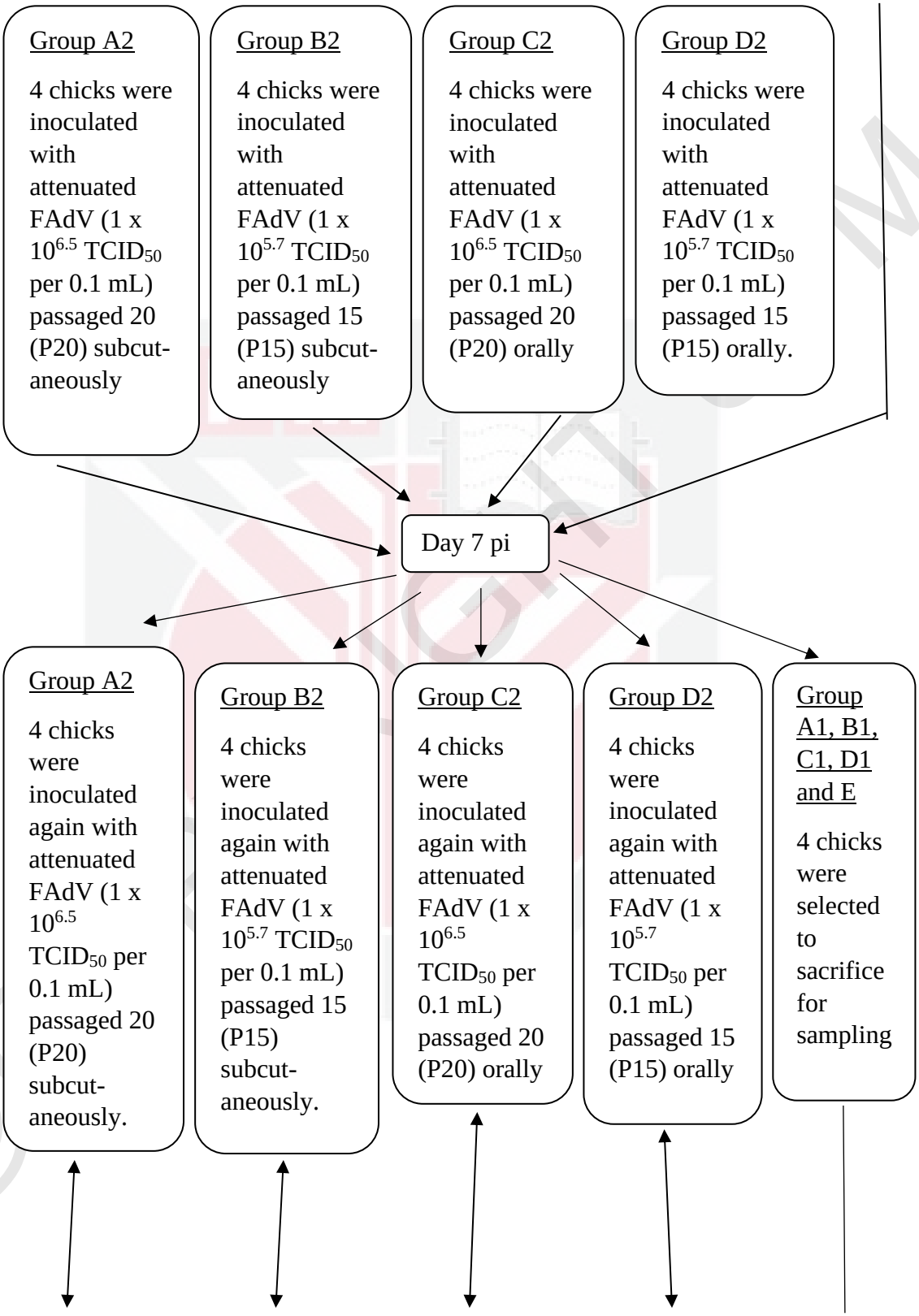
Uusikerttula H., Hulincurtis S., Davies J. and A.L. Parker (2015). Oncolytic adenovirus: Strategies and insights for vector design and immuno-oncolytic applications. *Viruses.* 7:6009–6042. doi: 10.3390/v7112923.

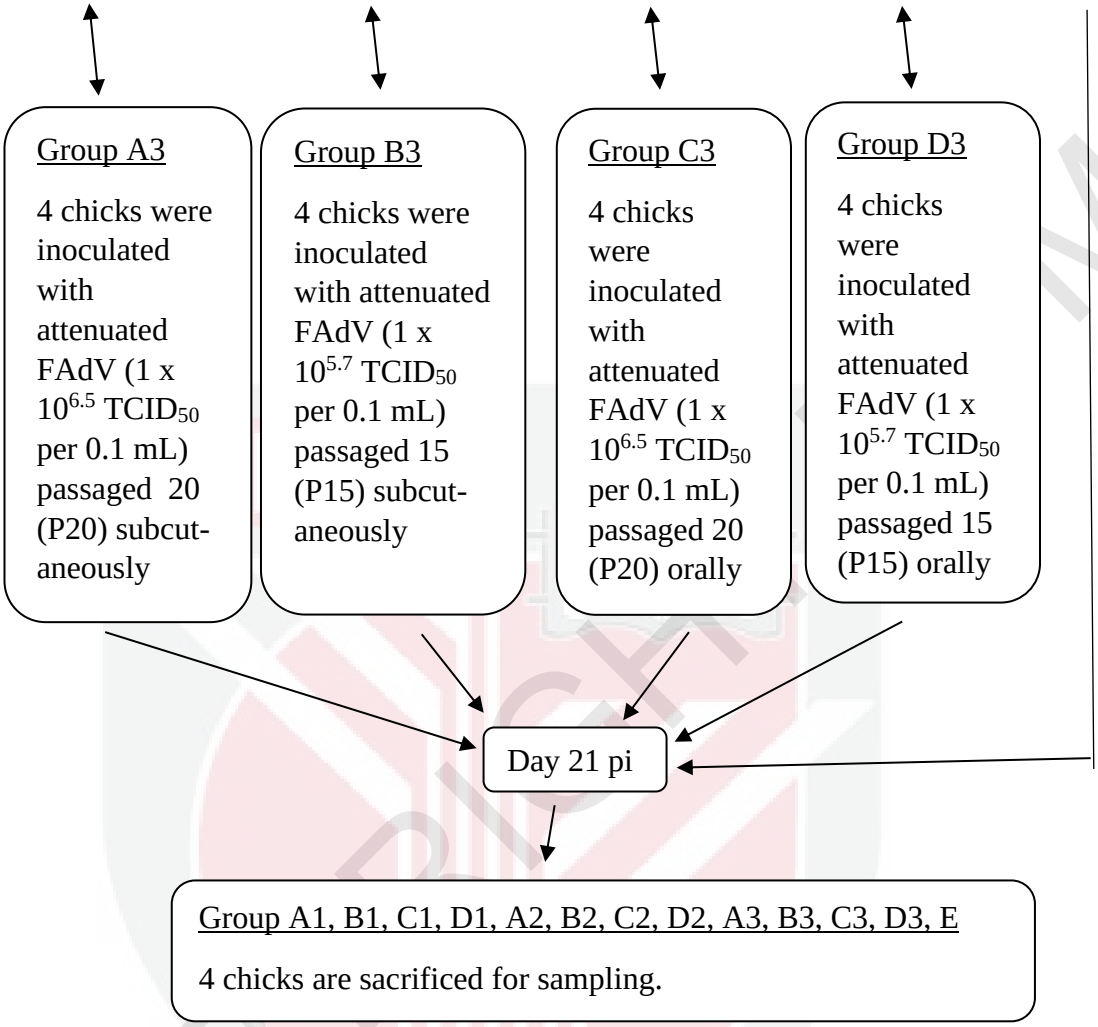
APPENDIX 1

Experimental design for effect of effect of different passages, times, and routes of inoculation on humoral immune response induced by attenuated fowl adenovirus in broiler chickens.




Group A1
8 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) subcut-aneously.
Group B1
8 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) subcut-aneously
Group C1
8 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) orally
Group D1
8 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) orally.
Group E
12 chicks were left un-inoculated without any FAdV and act as control group. 4 chicks are selected to sacrifice for sampling.
Group A2
4 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) subcut-aneously
Group B2
4 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) subcut-aneously
Group C2
4 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) orally
Group D2
4 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) orally.


Group A2
4 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) subcut-aneously
Group B2
4 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) subcut-aneously
Group C2
4 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) orally
Group D2
4 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) orally.
Day 7 pi
Group A2
4 chicks were inoculated again with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) subcut-aneously.
Group B2
4 chicks were inoculated again with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) subcut-aneously.
Group C2
4 chicks were inoculated again with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) orally
Group D2
4 chicks were inoculated again with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) orally
Group A1, B1, C1, D1 and E
4 chicks were selected to sacrifice for sampling


Group A3
4 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) subcut-aneously
Group B3
4 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) subcut-aneously
Group C3
4 chicks were inoculated with attenuated FAdV (1 x $10^{6.5}$ $TCID_{50}$ per 0.1 mL) passaged 20 (P20) orally
Group D3
4 chicks were inoculated with attenuated FAdV (1 x $10^{5.7}$ $TCID_{50}$ per 0.1 mL) passaged 15 (P15) orally
Day 21 pi
Group A1, B1, C1, D1, A2, B2, C2, D2, A3, B3, C3, D3, E
4 chicks are sacrificed for sampling.

APPENDIX 2

Table 1: Body weight of chickens throughout the trial.

Day pi	Body Weight (Mean ± SEM g)				
	Subgroup 1				Group E (Control)
	Group A1	Group B1	Group C1	Group D1	
0	67.4±3.3[a]				
7	261.8±16.4[b]	251.5±22.7[b]	279.0±8.5[b]	243.3±20.1[b]	250.0±13.0[b]
21	1096.5±38.6[ch]	1030.8±20.5[gh]	1411.8±43.8[e]	1171.5±34.0[cdfg]	964.0±23.5[h]
	Subgroup 2				
	Group A2	Group B2	Group C2	Group D2	
21	1116.3±58.1[ch]	1071.8±48.5[dh]	1276.3±35.5[ce]	1228.8±57.2[cdef]	
	Subgroup 3				
	Group A3	Group B3	Group C3	Group D3	
21	1102.5±27.8[ch]	1110.5±21.2[ch]	1244.5±41.5[cde]	1250.5±27.9[cdef]	

Each value is the mean ± standard error of mean of 4 chickens from each group.

[a-b-c-d-e-f-g-h] means within the row had common superscripts differs at $p<0.05$. Mean with different superscript had difference between body weight within the same age group (across row).

APPENDIX 3

Table 2: Liver weight of chickens throughout the trial.

Day pi	Liver Weight (mean ± SEM g)				
	Subgroup 1				Group E (Control)
	Group A1	Group B1	Group C1	Group D1	
0	2.9±0.1[a]				
7	9.5±0.7[b]	10.0±0.7[b]	9.5±0.5[b]	9.0±9.8[b]	10.0±0.7[b]
21	23.0±1.7[c]	18.8±1.0[c]	25.8±1.7[c]	23.5±0.7[c]	19.8±1.8[c]
	Subgroup 2				
	Group A2	Group B2	Group C2	Group D2	
21	19.5±1.2[c]	19.3±0.3[c]	22.3±1.0[c]	20.0±0.4[c]	
	Subgroup 3				
	Group A3	Group B3	Group C3	Group D3	
21	21.3±1.5[c]	20.3±1.3[c]	22.5±0.7[c]	20.0±1.4[c]	

Each value is the mean ± standard error of mean of 4 chickens from each group.

[a-b-c] means within row with no common superscripts differs at $p<0.05$.

APPENDIX 4

Table 3: Liver to body weight ratio of chickens throughout the trial.

Day pi	Liver weight : body weight (Mean ± SEM g) 10^{-2}				
	Subgroup 1				Group E (Control)
	Group A1	Group B1	Group C1	Group D1	
0	4.29±0.16[a]				
7	3.63±0.07[b]	4.02±0.25[b]	3.40±0.09[b]	4.00±0.23[b]	4.00±0.23[b]
21	2.09±0.11[c]	1.82±0.10[c]	1.83±0.13[c]	2.01±0.07[c]	2.04±0.13[c]
	Subgroup 2				
	Group A	Group B	Group C	Group D	
21	1.75±0.07[c]	1.80±0.05[c]	1.75±0.11[c]	1.63±0.05[c]	
	Subgroup 3				
	Group A	Group B	Group C	Group D	
21	1.92±0.09[c]	1.82±0.09[c]	1.81±0.03[c]	1.59±0.08[c]	

Each value is the mean ± standard error of mean of 4 chickens from each group.

[a-b-c] means within row with no common superscripts differs at $p<0.05$.

APPENDIX 5

Table 4: FAdV antibody titer of chickens throughout the trial.

Day pi	FAdV Antibody (Mean ± SEM ELISA unit)				
	Subgroup 1				Group E (Control)
	Group A1	Group B1	Group C1	Group D1	
0	5672±1275[a]				
7	380±99[b]	771±289[b]	1557±528[b]	895±506[b]	1256±730[b]
21	486±362[b]	567±277[b]	347±91[b]	231±94[b]	236±88[b]
	Subgroup 2				
	Group A2	Group B2	Group C2	Group D2	
21	914±367[b]	1956±916[b]	1003±566[b]	958±492[b]	
	Subgroup 3				
	Group A3	Group B3	Group C3	Group D3	
21	969±420[b]	1383±541[b]	445±166[b]	436±310[b]	

Each value is the mean ± standard error of mean of 4 chickens from each group.

[a-b] means within row with no common superscripts differs at $p<0.05$.