UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY AND IMMUNOGENICITY OF FOWL ADENOVIRUS ATTENUATED IN CHICKEN EMBRYO LIVER CELLS IN SPECIFIC PATHOGEN FREE CHICKENS

NURUL AYUNI BINTI AZMI

PATHOGENICITY AND IMMUNOGENICITY OF FOWL ADENOVIRUS ATTENUATED IN CHICKEN EMBRYO LIVER CELLS IN SPECIFIC PATHOGEN FREE CHICKENS

NURUL AYUNI BINTI AZMI

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

March, 2017

It is hereby certified that we have read this project paper entitled “Pathogenicity And Immunogenicity of Fowl Adenovirus Attenuated In Chicken Embryo Liver Cells In Specific Pathogen Free Chickens”, by Nurul Ayuni Binti Azmi and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

PROFESSOR DR. MOHD HAIR BIN BEJO

DVM (UPM), PhD (Liverpool, UK)

Professor

Faculty of Veterinary Medicine

Universiti Putra Malysia

Serdang, Selangor

(Supervisor)

ACKNOWLEDGEMENTS

First of all, I would like to send my heartiest gratitude to Allah S.W.T for allowing me to complete my final year project successfully. My deepest appreciation goes to my project supervisor, Professor Dr. Mohd Hair Bin Bejo for his sincere guidance and support throughout this project.

Next, I would like to express my gratitude to Dr. Norfitriah for helping me a lot in my project and also assisting me in my laboratory work. To the serology laboratory staff, especially Encik Saipuzaman and also the Animal Research Facility staff, thank you for all your kindness.

A big thanks to my final year project mates; Arif Izuan and Chong Zheng Zhe for their help during this project. Thank you for your time. I really enjoyed doing this project with you guys.

Not forgetting my parents; Azmi and Nor Zainab, my siblings; Ashraf, Aina and Kak Lea, my cutie pie; Uzma, Umar and Hana- thanks for always being there for me in my joy and sorrow. A dedication for my academic supervisor, Dr. Rozaihan for her support and motivation since I was in my first year of DVM.

Last but not least, I would like to acknowledge all of my beloved friends for their warm support and love. To everyone who helped me directly or indirectly, thank you so much and only God can repay your kindness.

CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

FAdV	Fowl Adenovirus
IBH	Inclusion Body Hepatitis
CEL	Chicken Embryo Liver
SPF	Specific Pathogen Free
P15	Passage 15
P20	Passage 20
P25	Passage 25
ELISA	Enzyme-linked Immunoabsorbent Assay
pi	Post Inoculation
IBD	Infectious Bursal Disease
CAV	Chicken Anemia Virus
INIBs	Intranuclear Inclusion Bodies
TEM	Transmission Electron Microscope
PCRF	Pairwise Comigrating Restriction Fragment
PBLs	Peripheral Blood Lymphocytes
PHA-P	Phytohemagglutinin

ABSTRAK

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

PATOGENISITI DAN IMUNOGENISITI ADENOVIRUS AVIAN YANG DILEMAHKAN DALAM SEL HATI EMBRIO AYAM DALAM AYAM BEBAS PATOGEN KHUSUS

Oleh

Nurul Ayuni Binti Azmi

Mac 2017

Penyelia : Profesor Dr. Mohd Hair Bin Bejo

Jangkitan adenovirus avian (FAdV) telah tersebar ke seluruh dunia dan seringkali dikaitkan dengan jangkitan inklusi badan hepatitis (IBH). Penyakit ini boleh menyebabkan kadar kematian yang tinggi dalam ternakan ayam. Oleh itu, objektif kajian ini adalah untuk menentukan patogenisiti dan imunogenisiti FAdV isolat (UPM1137) yang telah dilemahkan dalam sel hati embrio ayam (CEL) pada ayam bebas pathogen khusus (SPF). Empat puluh lima anak ayam telah dibahagikan kepada empat kumpulan iaitu A, B, C dan D. Ayam dari kumpulan A, B dan C telah disuntik dengan 0.1mL FAdV berlainan pasaj iaitu pasaj 15 (P15) dengan $10^{5.2}$ $TCID_{50}$/0.1mL titer virus, 20 (P20) dengan $10^{5.6}$ $TCID_{50}$/0.1mL titer virus dan 25 (P25) dengan $10^{5.2}$ $TCID_{50}$/0.1mL titer virus, melalui laluan subkutis pada umur 1

dan 14 hari. Kumpulan D dijadikan sebagai kumpulan kawalan dan kekal tidak disuntik. Pensampelan dijalankan pada umur anak ayam 1, 14 dan 28 hari. Anak ayam tersebut diperhatikan sebanyak dua kali sehari bagi sebarang perubahan klinikal. Sampel darah dan berat badan juga berat hati diambil dan direkodkan. Sampel hati diambil dan diawet dalam 10% bufer formalin untuk pemeriksaan histologi. Kajian ini menunjukkan bahawa tiada sebarang tanda klinikal yang tidak normal pada kesemua ayam sepanjang tempoh kajian. Tiada lesi mata kasar dan histologi dijumpai pada hati ayam. Ujian ELISA mendapati titer antibodi FAdV tidak dapat dikesan dalam kesemua ayam kumpulan D (Kawalan). Walaubagaimanapun, titer antibodi mula dikesan pada hari ke 14 dalam kesemua kumpulan ayam yang disuntik dan antibodi terus meningkat pada hari ke 28 di mana ia menunjukkan perbezaan bererti ($p<0.05$) apabila dibandingkan dengan kumpulan D (Kawalan). Kesimpulannya, FAdV yang telah dilemahkan dalam sel hati embrio ayam dalam kajian ini adalah rendah patogenik dan berupaya mendorong penghasilan FAdV antibodi dalam ayam SPF.

Kata kunci: Adenovirus ayam (FAdV), ayam bebas pathogen khusus (SPF), laluan subkutis, pemeriksaan histologi.

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 – Project.

PATHOGENICITY AND IMMUNOGENICITY OF FOWL ADENOVIRUS ATTENUATED IN CHICKEN EMBRYO LIVER CELLS IN SPECIFIC PATHOGEN FREE CHICKENS

By

Nurul Ayuni binti Azmi

2017

Supervisor: Professor Dr. Mohd Hair bin Bejo

Fowl adenovirus (FAdV) infection is distributed worldwide and is associated with inclusion body hepatitis (IBH). This disease can cause high mortality rate in flocks of chicken. Thus, it was the objective of this study to determine the pathogenicity and immunogenicity of FAdV isolate (UPM1137) attenuated live in chicken embryo liver (CEL) cells in specific pathogen free (SPF) chickens. Forty five one-day-old SPF chicks were divided into four groups namely A, B, C, and D. Group A, B and C were inoculated with 0.1mL of FAdV passage 15 (P15) with virus titre of $10^{5.2}$ $TCID_{50}$/0.1mL, 20 (P20) with virus titre of $10^{5.6}$ $TCID_{50}$/0.1mL and 25 (P25) with virus titre of $10^{5.2}$ $TCID_{50}$/0.1mL, respectively through subcutaneous route on days 1 and 14 of age. The group D acted as control group and remain non-inoculated. Sampling was done on days 1, 14 and 28 of age. The chicks were observed at least

twice daily for any clinical sign abnormality. Prior to necropsy, blood was collected, and body and liver weight were recorded. Sample of liver was fixed in 10% buffered formalin for histological examination. The study revealed that there were no abnormal clinical signs in all chickens throughout the trial. There were also no significant gross and histological lesions in the liver. The ELISA results showed that FAdV antibody titer was not detected in group D (Control). However, the antibody titer was induced at day 14 in all inoculated group and continued to increase at day 28 of age which is significantly different ($p<0.05$) when compared to the group D (Control). It was concluded that the live attenuated FAdV in CEL cells is low pathogenic and could induce high FAdV antibody in SPF chickens.

Keywords: Fowl adenovirus (FAdV), specific pathogen free (SPF) chickens, subcutaneous route, histological examination.

1.0 INTRODUCTION

1.1 Background of the Study

Fowl adenovirus (FAdV) is a non-enveloped, icosahedral, empty and complete virions with a diameter of 70-85 nm (Ganesh *et al.*, 2002). According to Adair and Fitzgerald (2008), avian adenovirus was first isolated from diseased birds from an outbreak of respiratory disease and called adenoidal-pharyngeal-conjunctival agents before changing to adenovirus.

FAdVs that infect chickens are from subgroup I. It was classified in the genus of *Aviadenovirus* and under the family of Adenoviridae before further classified into five species and 12 serotypes (Benko *et al*., 2000). Mcferran *et al.* (1972) mentioned that this 12 serotypes are varies in pathogenicities and can be isolated from both healthy and sick birds. FAdV is the aetiology for naturally acquired outbreaks of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome, respiratory tract disease and gizzard erosions in birds which can contributes to economic loss (Alvarado *et al*., 2007; Grimes *et al*., 1977; Nakamura *et al*., 1999; Mase *et al*., 2009; Ono *et al*., 2004).

Adair and Fitzgerald (2008) stated that IBH affects mostly broilers between the age of 3-7 weeks but there was also cases recorded as young as 7 days and as old as 20 weeks. It causes a sudden onset of mortality which peaks after 3-4 days of infection and usually stops on day 5 but sometimes continue for 2-3 weeks. They also revealed that sick birds show signs of crouching position with ruffled feathers and probably die within 48 hours or recover. Morbidity of this disease is low but mortality can achieve 10% or as high as 30% but this occurs occasionally.

FAdV can be transmitted vertically through embryonated egg or horizontally by direct fecal contact of infected birds and also from fomites (Hair-Bejo, 2005). At first, it was believed that IBH can only occur if the immune system of the birds already weakened by other immunosuppressive agents such as infectious bursal disease (IBD), chicken infectious anemia or mycotoxicosis. Until a recent study revealed that virulent strains had emerged and can cause severe disease alone leading to mortality ranging from 10-30% (Gaba *et al*., 2010; Dahiya *et al*., 2002)

IBH was first described in USA in the year of 1963 and then distributed all over the world (Helmboldt and Frazier, 1963). Hair-Bejo (2005) was first to report the disease occurrence in Malaysia. He further explained that the role of IBH is little known but the effects of high mortality and poor performance due to the outbreak will gives a significant impact on poultry industry. A recent study proved that Malaysian FAdV isolate (UPM04217) from recent field outbreak are pathogenic in Specific Pathogen Free (SPF) chickens eggs with 100% mortality but showed low pathogenicity in SPF chicken since no clinical signs, mortality and gross lesions were found (Alemnesh *et al*., 2012).

The main lesions that can be seen in IBH are pale, friable and swollen livers with petechial or ecchymotic hemorrhages that might be present in the liver and skeletal muscles. In degenerated hepatocytes, basophilic or eosinophilic intranuclear inclusions bodies (INIBs) can be observed. In a study by Riddell (1987), Basophilic INIBs in hepatocytes of IBH infected birds contain quite an amount of adenoviruses under transmission electron microscope (TEM). Meanwhile, there is only fibrillar granular material and filaments in the eosinophilic INIBs. Saifuddin and Wilks (1992)

mentioned that a virulent strain of adenovirus which was isolated from an infected broiler with IBH results in severe lymphocytic depletion in bursa, thymus and spleen of commercial broiler and SPF chickens. Virus isolation can be done by taking liver sample infected with IBH (Adair and Fitzgerald, 2008).

1.2 Hypothesis

The hypothesis of the study were:

1. FAdV attenuated in CEL cells is low pathogenic and could not induce high FAdV antibody in SPF chickens.
2. FAdV attenuated in CEL cells is low pathogenic and could induce high FAdV antibody in SPF chickens.

1.3 Objectives

The objectives of this study were:

1. to determine the pathogenicity of FAdV attenuated in CEL cells in SPF chickens.
2. to determine the immunogenicity of FAdV attenuated in CEL cells in SPF chickens.

2.0 LITERATURE REVIEW

2.1 Aetiology

2.1.1 Classification

Fowl adenovirus (FAdV) is classified under family of adenoviridae, genus of *Aviadenovirus* which only affect birds and further classified into five species (A to E) and 12 serotypes (Benko *et al*., 2000). According to Zsák and Kisary (1984), the species classification of FAdV is based on their restriction enzyme fragment patterns, phylogenetic relationships, pathogenicity, cross-neutralization and recombinant potential. Whereas, the serotypes are determined based on results of cross neutralization tests (Adair and Fitzgerald, 2008). Furthermore, Erny *et al.,* (1991) stated that the pathogenicity and genetic relationship is vary with different isolates of the same species and serogroups based on PCRF analysis. Therefore, there is a need to determine the types of local isolates to come out with an effective control and preventive measures to overcome this disease. In Malaysia, the dominant serotype of FAdV detected by molecular characterization is FAdV-8b which is responsible for inclusion body hepatitis outbreak in this country (Juliana *et al*., 2014)

2.1.2 Morphology

Adenovirus is a non-enveloped, icosahedral structure which consists of 240 non-vertex capsomers (hexon) and 12 vertex (pentons) each with a fiber protruding from the virion surface. However, Aviadenovirus has two fiber proteins per vertex (Benko *et al*., 2005). The virions size is about 70-90nm diameter and having density range of

between 1.32-1.37g/ml in cesium chloride and the nucleic acid is double-stranded DNA (Adair and Fitzgerald, 2008).

2.2 Epidemiology

2.2.1 Mode of Transmission

Both vertical and horizontal transmission is involved in spreading of the virus. However, vertical transmission is more important where disease can be transmitted through the embryonated egg. Horizontal transmission can occur through fomites, personnel and also transport. Virus are present at high titres in the feces (Alemnesh *et al*., 2011). According to Adair and Fitzgerald (2008), the peak excretion of virus in broilers is between 4-6 weeks, meanwhile for layer it was from 5-9 weeks after infection. Besides that, they also explained that adenovirus can become latent and untraceable for at least one generation. If young chicks come in contact with IBH infected chicks, they can die of per acute infection (Rahimi and Haghighi, 2015).

2.2.2 Pathogenesis

Adenoviruses are found universally in chickens based on the serological and virological studies and the virus can also be isolated from healthy and sick birds (Mcferran and Smyth, 2000). FAdV first started to multiply in the small and large intestine once it entered the body. In the early phase of virus replication, after the virus entered the host cell the virus DNA will be transferred to the nucleus and transcription and translation of the genes will take place (Russell, 2009). It was then circulate in the body and attack vital organs such as liver, kidney, respiratory tract, bone marrow and bursa which causes viraemia (Anjum, 1990; Toro *et al*., 2000). FAdV can be isolated

from the feces, trachea and targeted organs for example liver and gizzard, but in IBH cases liver is more important (Adair and Fitzgerald, 2008). However, high titre of virus can be found in the feces (Alemnesh *et al*., 2012). Anjum (1990) also explained that chicken that was affected with adenovirus will become carrier throughout his life.

2.3 Clinical Signs and Pathological Lesions

The important signs of IBH which is caused by FAdV includes the history of acute infection and sudden death which peaks after 3-4 days of infection but usually ends at day five post infection and rarely the infection continues for 2-3 weeks (Hair-Bejo, 2005). It is usually seen in broiler chickens of 3 to 7 weeks. Other than that, McFerran and Smyth (2000) stated that clinical signs such as crouching position, ruffled feathers and fatality or recovery within 48 hours can be observed. Although mortality rate is high, but the morbidity of this disease is still low.

Upon necropsy of affected chicken, the gross lesions that can be seen includes pale, swollen and friable liver with petechial or ecchymotic haemorrhages if present. Besides that, haemorrhages can also be seen on skeletal muscles. For the histopathological lesions, the classical signs of IBH is the inclusion bodies which can be found on the hepatocytes. It can be either eosinophilic large, round or irregular shaped with a clear pale halo or rarely basophilic (Adair and Fitzgerald, 2008). McFerran and Smyth (2000) in their study stated that FAdV can be found in the basophilic inclusions whereas the eosinophilic inclusions only consists of fibrillar granular material. They also added that in natural cases of IBH, necrotising pancreatitis and gizzard erosions and/or ulceration can be present but there was no

inclusion bodies detected in the gizzard. Besides that, Hair-Bejo (2005) mentioned that there was a moderate degeneration and necrotisation of the hepatic parenchyma.

2.4 Comparison between Live Attenuated and Killed FAdV Vaccine

According to Khan *et al.* (2005), vaccine formulations that is being used in the field currently does not provide a long term immune response against the FAdV diseases. They also added that killed vaccine is not efficient. Killed vaccine by using liver homogenates inactivated by formalin is widely used (Mansoor *et al*., 2011). Therefore, a need of local and attenuated contained vaccine is needed for an efficient protection. As studied by Mansoor *et al.* (2011), the 16th passage of the virus is non-pathogenic to the day-old-chicks. In terms of antibody titers, seroconversion can be detected as early as 1-week post inoculation (pi). The antibody titers at day 7, 14 and 21 post vaccination from the live-attenuated vaccine shows increment and higher than induced by killed vaccine. However, antibody titers from the killed vaccine increase at day 7 and 14 but then decrease on day 21 post vaccination. Furthermore, live attenuated embryo-adapted FAdV-4 vaccine attempts to produce higher antibody response compared to chicken vaccinated with killed vaccine. This proves by the maximum protection of 95% with low morbidity, mortality and pathological lesions in the chicken provided by the live attenuated vaccine after challenge shot had been given as compared to the killed vaccine which only gives 55% of protection. Higher antibody response throughout the study shows the primacy of the live attenuated vaccine. Other than that, the advantage of live attenuated compared to killed vaccine is that it is more resistant to challenge. A developed live attenuated vaccine should be on field trials for several times before commercially produced.

Mansoor *et al*., (2011) also mentioned that the inability of killed vaccine to become efficient is due to chemical reaction probably from the formalin with the surface proteins and then altering the surface proteins of the virus. This process diminished the immune response produced by the body.

2.5 Antibody Response

Enzyme-linked Immunoabsorbent Assay (ELISA) is a sensitive test to evaluate the humoral immune response of chickens infected with FAdV. It can detect antibody as early as 7 days pi. Thus, it is more preferred to be used for early detection of infection (Maiti and Sarkar, 1997). In a study by Singh *et al.* (2006), it had been proven that both cell mediated and humoral immune response are affected by immunosuppression caused by FAdV. FAdV causes reduction in mitogenic response of peripheral blood lymphocytes (PBLs) of infected chicks to phytohaemagglutinin (PHA-P) stimulation on week two and three post infection. On the other hand, humoral immunosuppressive effect is shown by lowered IgM responses with unaffected IgG responses, reduced serological responses to other antigens and vaccine and also reduction in bursal index on week two post infection.

In another study by Lal *et al*., (1991), it focused on the cellular mediated immunity response as it also contributes in protection against viral infection other than humoral immunity. Transient cellular immune response which is induced by the virus causing this type of immunity to occur as early as one week pi and is maintained to the next five week. The injection of mitogen acts as in vivo assay to cellular immunity. The PHA-P response is statistically significant only at two weeks of pi. This response is proportional to the number of circulating T-lymphocytes. After five weeks of pi, the

PHA-P response will be reduced. This supports the previous mentioned study by Singh *et al*., (2006).

2.6 Diagnosis

2.6.1 Classical Classification

There are several classical ways to diagnose FAdVs. This includes examination of gross and histopathological changes in the liver, virus isolation in cell cultures, staining, electron microscopy and serological typing such as ELISA, agar gel immunodiffusion, neutralisation test and lastly hemagglutination test (Juliana *et al*., 2014)

The isolation of virus is important for the diagnostic procedure followed by further serotyping. This can help in determining local types of FAdV and production of vaccine in the future. Important specimens to be used includes feces, pharynx, kidney and affected organs. In cases of IBH liver sample should be taken for isolation. Primary cell culture that is preferred for FAdV isolation are chick embryo liver cells or chick kidney cells although there had been reported that Specific-Pathogen-Free embryonated chicken eggs also sensitive for FAdV cultivation.

2.6.2 Molecular Classification

Nucleic acid technology is more precise and reliable for classification of FAdVs compared to serological testing (Barua *et al*., 2005). This is because serological methods can lead to misinterpretation due to presence of antibodies against FAdVs.

Nowadays, some molecular identification and classification for FAdV typing such as by using PCR alone, PCR together with DNA sequencing or restriction enzymes analysis (REA) and a combination of these techniques had been used widely. These type of testing can also help in diagnosing mix infection of FAdV serotypes. However, there are several disadvantages of this method of classification such as long period of time to use, costly, and need an extensive interpretation (Juliana et al., 2014)

2.7 Control and Prevention

By applying effective biosecurity measures, horizontal transmissions can be prevented and vertical transmission can be avoided by controlling horizontal spread in commercial parent flocks (Juliana *et al*., 2014). Panigrahi (2016) mentioned that there should be isolation for the infected breeding flocks. As FAdVs infection can occur due to immunosuppression from IBDV and CAV, a proper control must be made to diminish these viruses. Vaccination is also one of the ways to prevent FAdVs diseases. Serotypes 4 and 8 are usually used for commercial vaccines production and commercial broilers are vaccinates at less than 10 days of age as their parents do not own serotype-specific adenovirus antibodies (Adair and Fitzgerald, 2008; Cowen, 1992). Alvorado *et al*., (2007) proven that broiler breeders can be protected by vaccinating them twice with autogenous killed vaccine against an experimental challenge of IBH due to the presence of maternal antibodies. Furthermore, adenoviruses is resistant to lipid solvents but can be inactivated with 1:1000 concentration of formaldehyde.

3.0 MATERIALS AND METHODS

3.1 FAdV Isolation

The FAdV isolate (UPM1137) was obtained from commercial layer chicken from a simultaneous outbreak of IBH and gizzard erosions in 2011. It was confirmed for FAdV by PCR and molecular characterization. Inoculum was obtained from virus supernatant in primary chicken embryo liver (CEL) cells from passage 15, 20 and 25 (UPM1137 CEL15, UPM1137 CEL20 and UPM1137 CEL25). All inoculums used were positive for FAdV by PCR and molecular characterization (Hair-Bejo, personal communication, 2017). The isolate serotype used was serotype 8b and was confirmed by sequence analysis and phylogenetic tree.

3.2 SPF Chicks

Day-old-chicks of SPF chickens were obtained from the Malaysian Vaccine Pharmaceutical (MVP) Sdn. Bhd., Puchong, Selangor.

3.3 Experimental Design

A total of 45 SPF day-old-chicks were divided into four groups namely groups A, B, C and D. The chicks in groups A, B and C were inoculated with 0.1mL of FAdV isolates passage 15 (P15) with virus titre of $10^{5.2}$ $TCID_{50}$/0.1mL, 20 (P20) with virus titre of $10^{5.6}$ $TCID_{50}$/0.1mL and 25 (P25) with virus titre of $10^{5.2}$ $TCID_{50}$/0.1mL, respectively through subcutaneous route. Meanwhile group D remain non-inoculated and acted as the control group. On day 1 of age, five chicks from control group were sacrificed by cervical dislocation for first sampling. Whereas the remaining chicks from the other groups were inoculated with the virus. The chicks were given feed and

water *ad-libitum* and monitored twice daily for any abnormal clinical signs. On day 14 of age, five chicks from each group were sacrificed and the remaining were inoculated with the same volume of FAdV inoculums. On day 28, all chicks (5) from each group were sacrificed for sampling. Body weight and blood samples were collected prior to necropsy. The gross lesions and liver weight were also recorded. Liver samples were fixed in 10% formalin for histological examination (Appendix 1).

3.4 Clinical Signs

Clinical signs were observed and recorded for any abnormalities twice a day.

3.5 Gross Lesions

Gross lesions were observed and recorded upon necropsy.

3.6 Histopathology

3.6.1 Hematoxylin and Eosin Stains

Samples of liver from the chicks were fixed in 10% buffered formalin for at least 24 hours. Then, the samples were trimmed to size of approximately 1cm x 1cm with thickness of 0.3cm before putting into the cassette. It was then processed and dehydrated in a series of alcohol and cleaned with xylene. After that, the sample was embedded in paraffin wax by using automated tissue processor. The tissue was then trimmed and sectioned about 4-5 with microtome and was mounted on the glass slide. The mounted tissue was dewaxed with xylene and 70%, 90% and 100% alcohol and stained with Hematoxylin and Eosin (HE) (Bancroft and Stevens , 1996). The slides

were viewed under microscope with x20, x40 and x100 objectives. Any histological changes were recorded.

3.7 Enzyme-linked Immunosorbent Assay

The serum samples were tested for FAdV antibody using commercial ELISA. Upon usage, the antigen coated plate was acclimatized to room temperature. After that, 100µL of negative and positive control were dispensed into respective wells accordingly. It was then followed by 100µL of 1:500 (v/v) diluted test sera into respective wells. The plate was covered and incubated at room temperature for 30 minutes. Next, it was washed 4 times with 300µL wash buffer per well. After washing, 100µL of sheep anti-chicken IgG labelled with alkaline phosphatase was added into the well. Then, it was further incubated for 30 minutes at room temperature. The plate was washed as mentioned above and 100µL of substrate buffer that contain diethanolamine buffer with enzyme co-factor was added into the well. Plate was incubated for next 15 minutes at room temperature. To stop the reaction, 100µL of stop solution was dispensed into each well. Lastly, microtitre plate reader was used to record the absorbance at 405nm and the FAdV antibody titer was then generated by using BioCheck 2000 software.

3.8 Statistical Analysis

Data was analysed by using IBM SPSS version 22 using parametric test which was one-way ANOVA to test for significant differences between groups (Ostertagová and Ostertag, 2013).

4.0 RESULTS

4.1 Clinical Signs

There were no abnormal clinical signs seen in all groups of chick from day 1 until day 28 of age (Fig., 1).

Figure 1: Normal condition of chicks in (a) group A and (b) group B on day 14 and (c) group C on day 28 of age.

4.2 Body Weight

Body weight of chickens were increased significantly from day 14 to day 28. However, there was no significant different ($p>0.05$) between groups on day 14 and day 28 respectively (Fig., 2 and Appendix, 2).

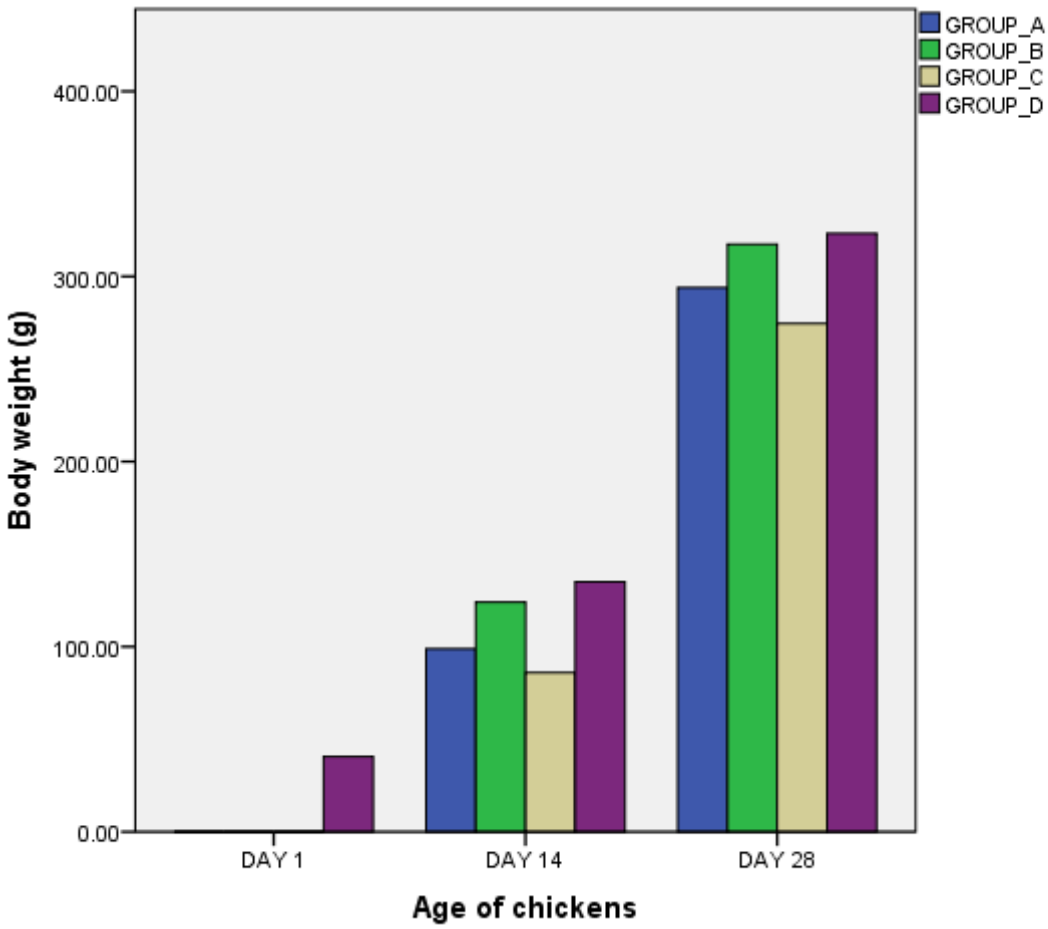


Figure 2: Body weight of chickens throughout the trial.

4.3 Liver Weight

On day 14, the liver weight was significantly different ($p<0.05$) between group A and C. Meanwhile, there was no significant different ($p>0.05$) between groups on day 28 of age (Fig., 3 and Appendix, 3).

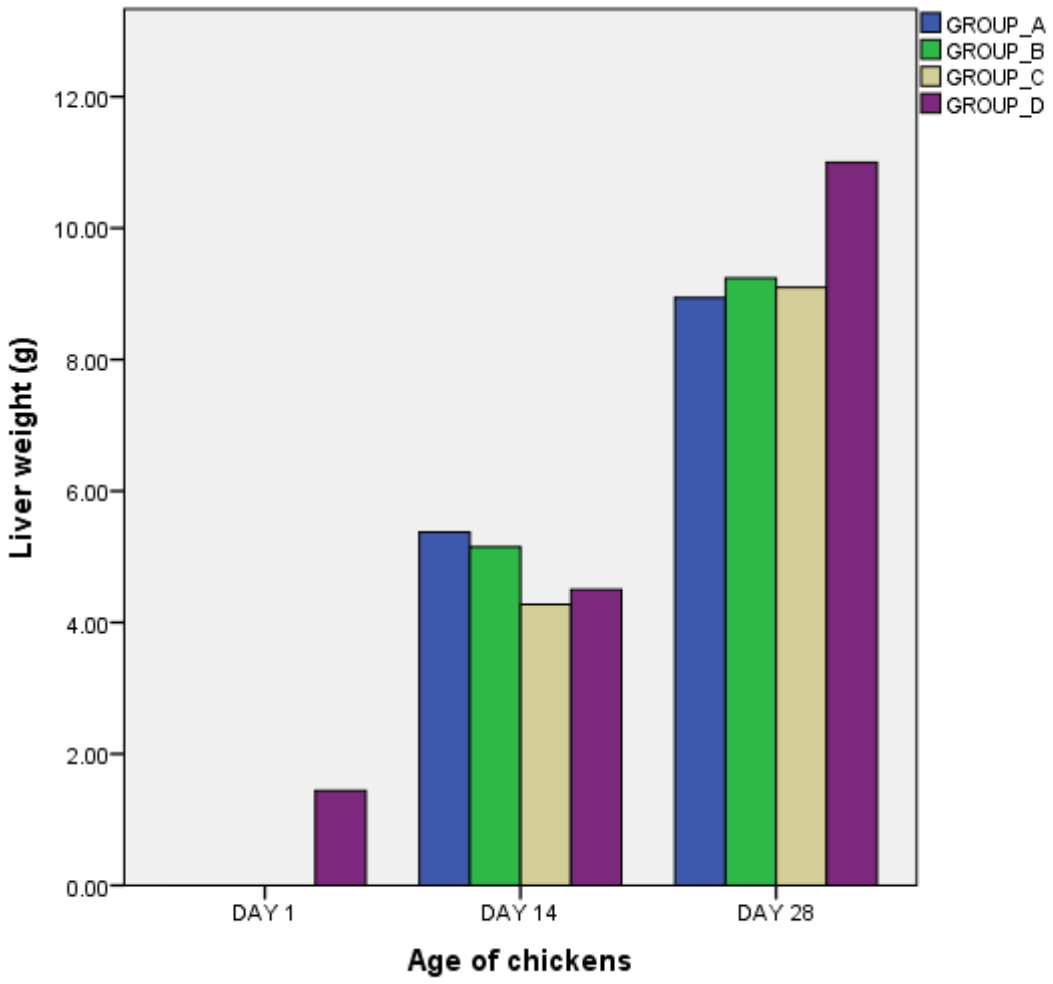


Figure 3: Liver weight of chickens throughout the trial.

4.4 Liver to Body Weight Ratio

Liver to body weight ratio was significantly different ($p<0.05$) on both days 14 and 28. The relative liver to body weight were significantly increased ($p<0.05$) in all of the inoculated groups on day 14 as compared to control group. In contrast, on day 28 the relative liver to body weight were significantly decreased in the inoculated groups when compared with the control group (Fig., 4 and Appendix, 4).

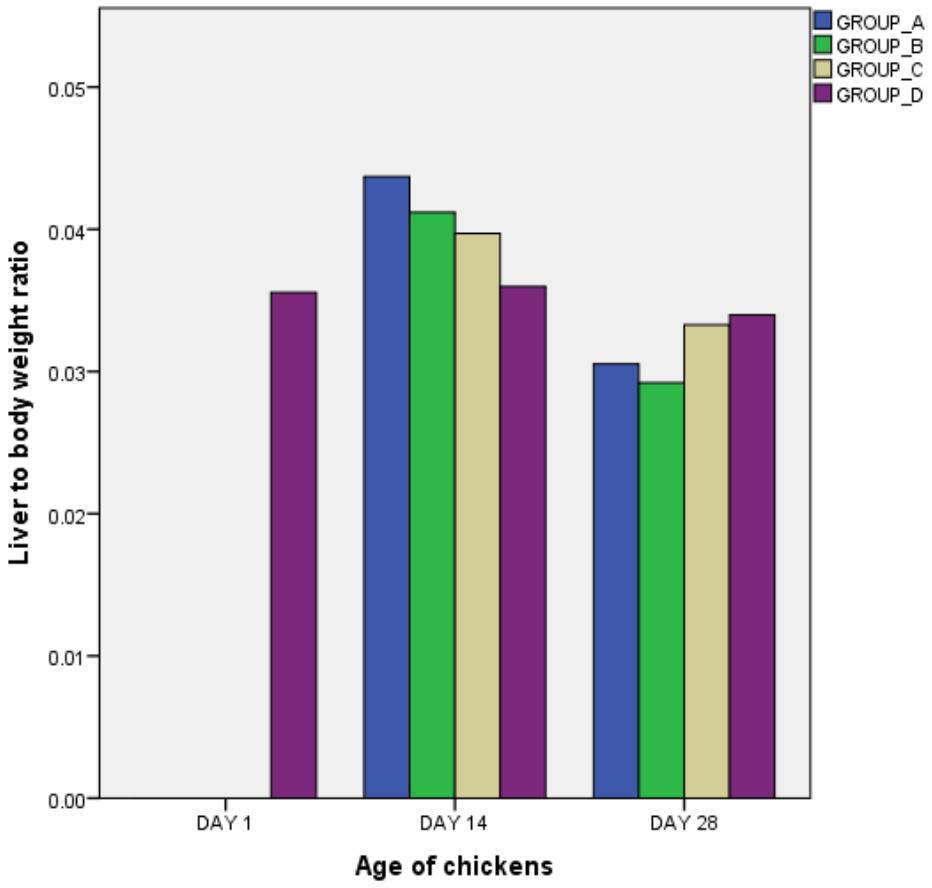


Figure 4: Liver weight of chickens throughout the trial.

4.5 Gross Lesions

4.5.1 Group A

The liver of all chicks remained normal throughout the experiment (Figs., 5a and 6a).

4.5.2 Group B

The liver of all chicks remained normal throughout the experiment (Figs., 5b and 6b).

4.5.3 Group C

The liver of all chicks remained normal throughout the experiment (Figs., 5c and 6c).

4.5.4 Group D

The liver of all chicks remained normal throughout the experiment (Figs., 5d and 6d).

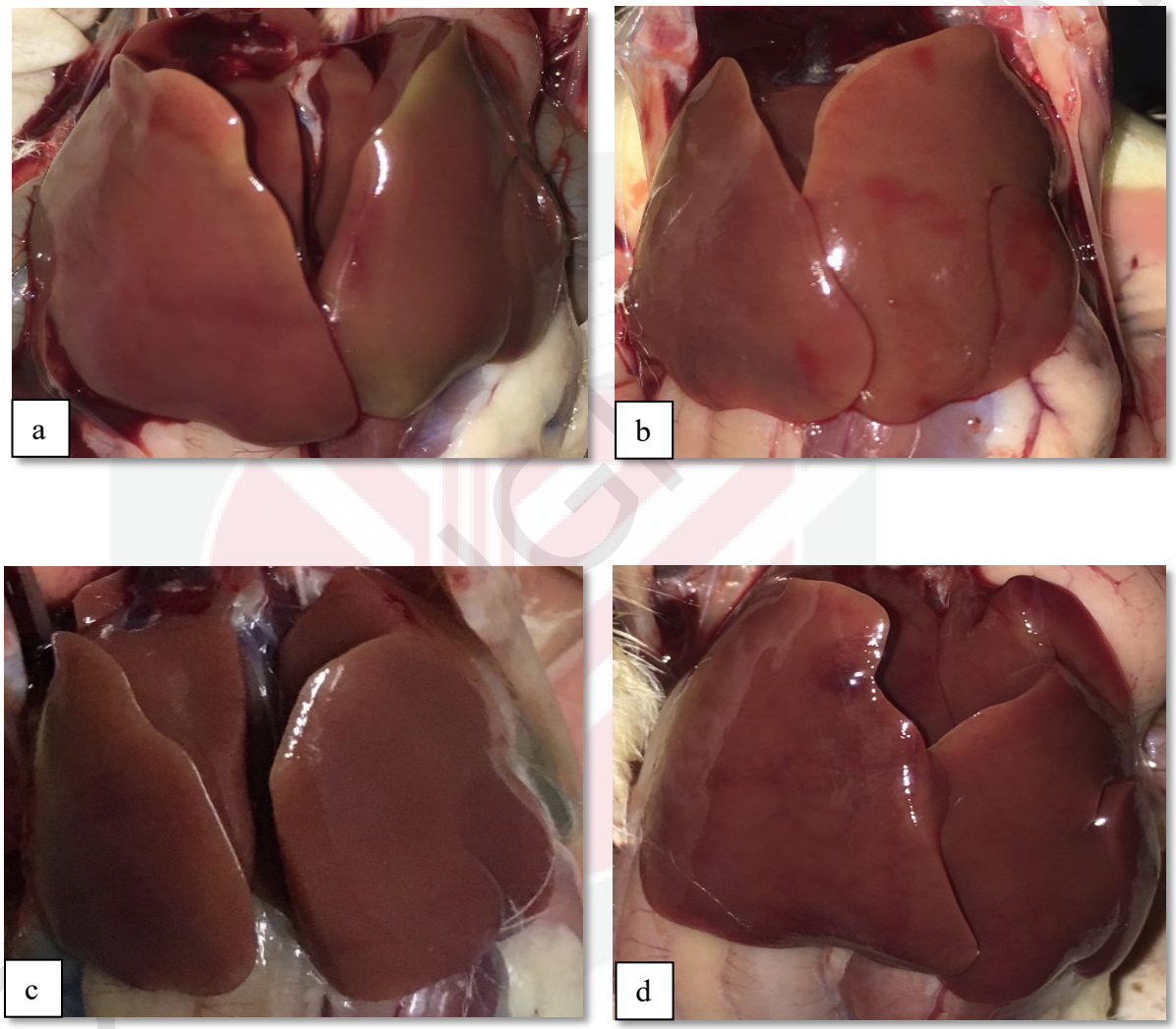


Figure 5: Normal liver of chicks in (a) group A, (b) group B, (c) group C and (d) group D on day 14 of age.

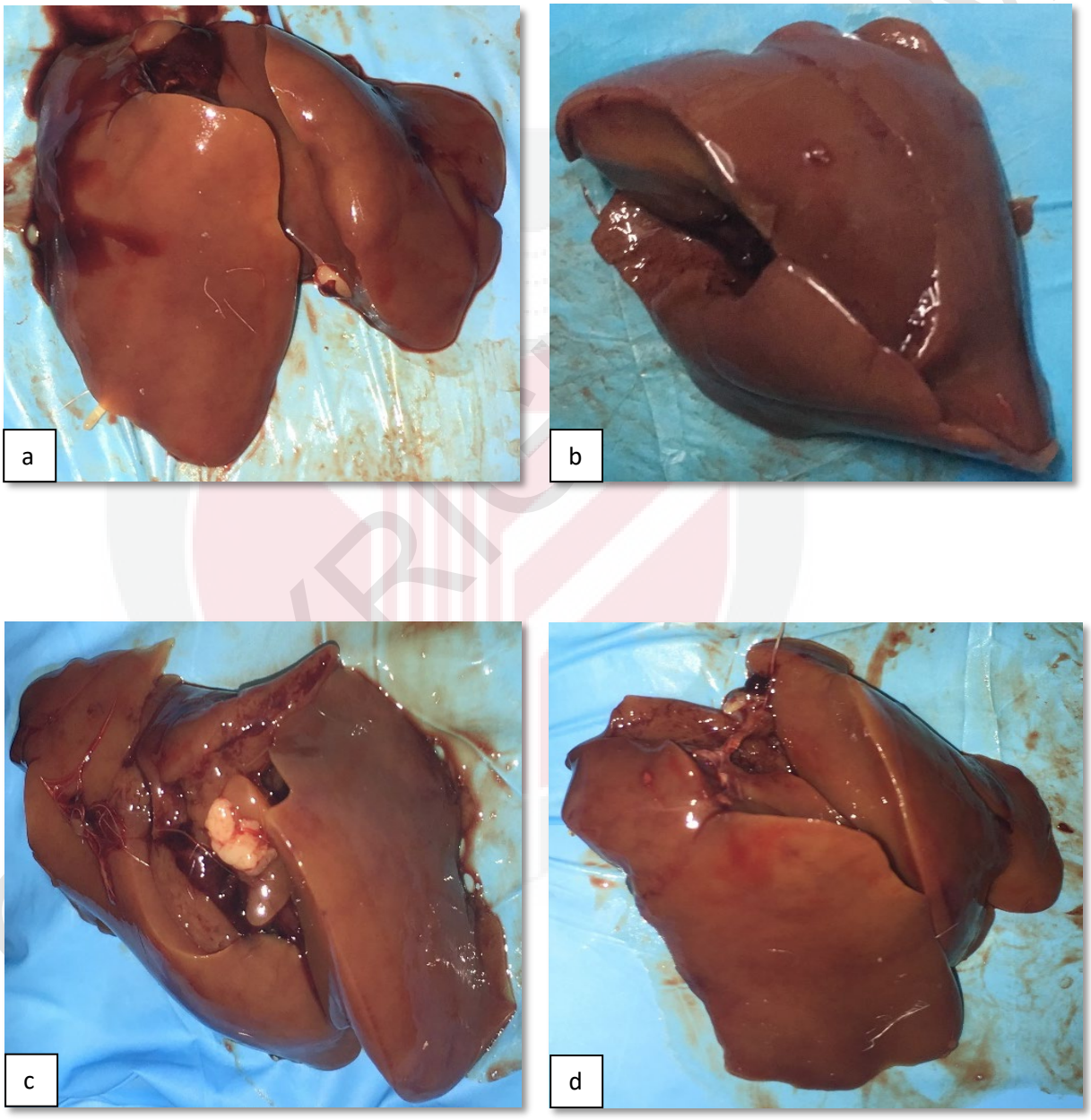


Figure 6: Normal liver of chicks in (a) group A, (b) group B, (c) group C and (d) group D on day 28 of age.

4.6 Histopathology

4.6.1 Group A

No significant findings throughout the trial (Figs., 7a and 8a).

4.6.2 Group B

No significant findings throughout the trial (Figs., 7b and 8b).

4.6.3 Group C

No significant findings throughout the trial (Figs., 7c and 8c).

4.6.4 Group D

No significant findings throughout the trial (Figs., 7d and 8d).

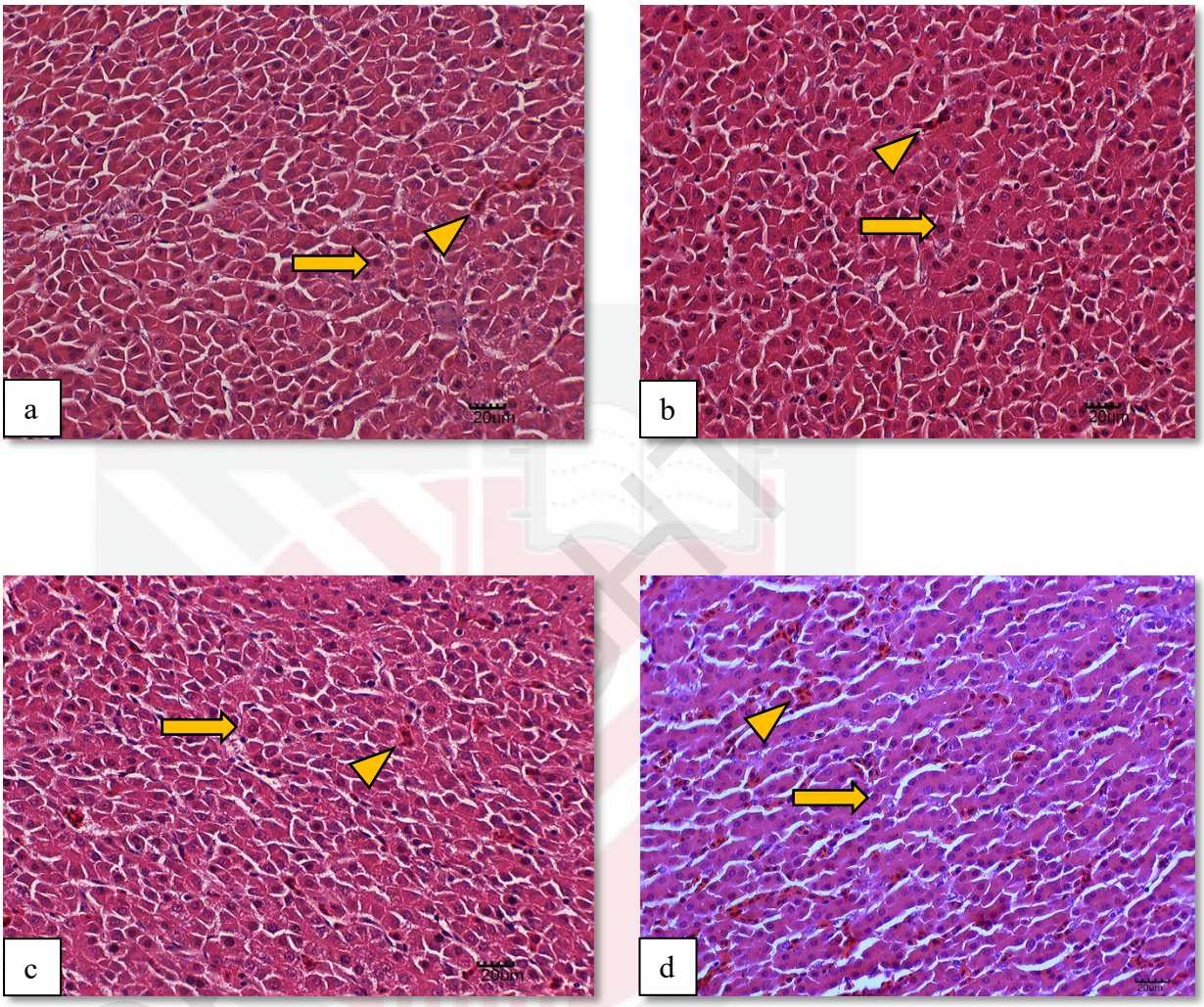


Figure 7: Hepatocytes of liver with normal size of nucleus (arrows) from chicks in (a) group A, (b) group B, (c) group C and (d) group D on day 14 of age. Note the presence of red blood cells in the sinusoids (arrow heads). Bar=20µm, HE

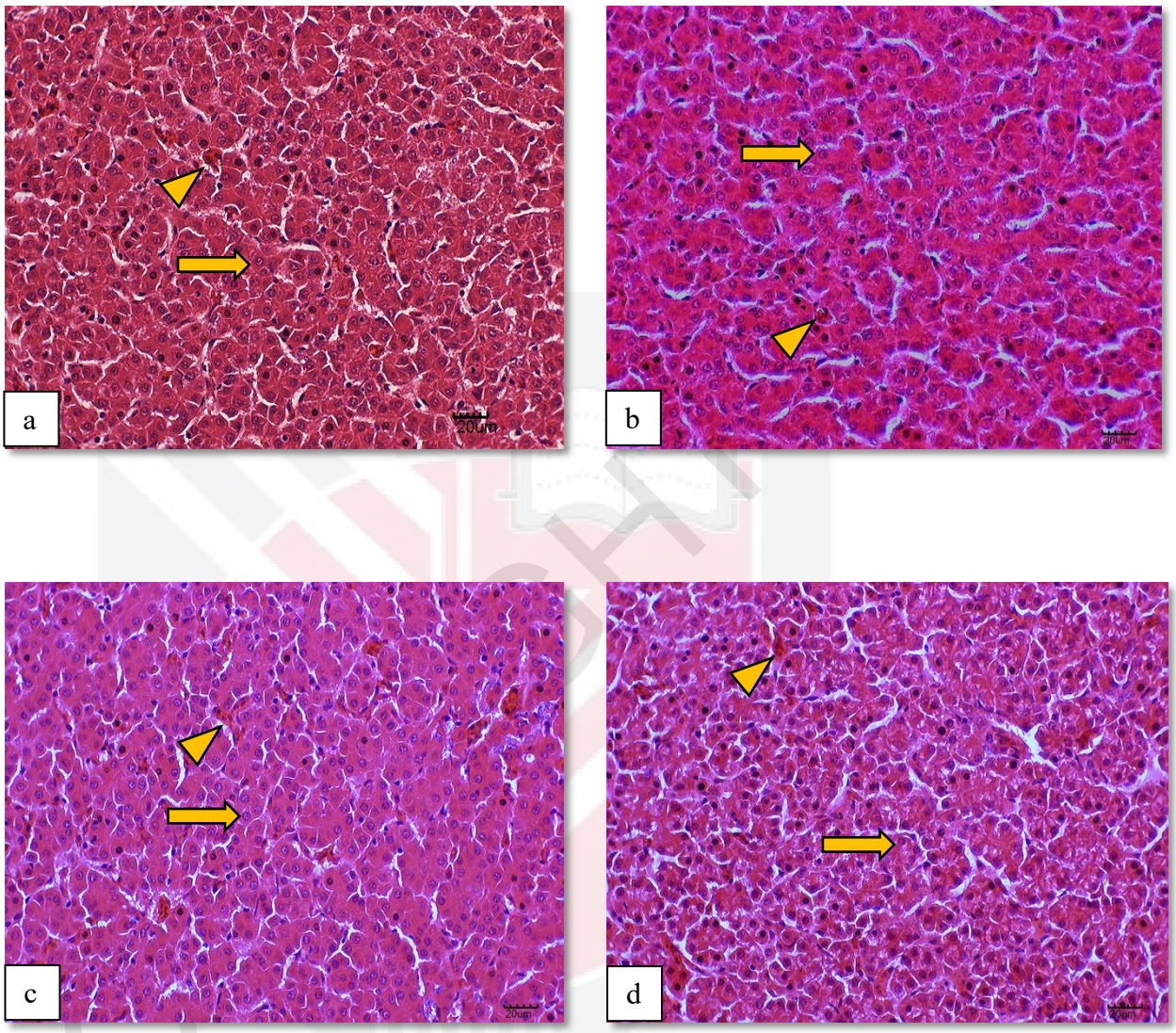


Figure 8: Hepatocytes of liver with normal size of nucleus (arrows) from chicks in (a) group A, (b) group B, (c) group C and (d) group D on day 28 of age. Note the presence of red blood cells in the sinusoids (arrow heads). Bar=20µm, HE

4.7 Antibody Titer

Antibody titers were not detected for control group on days 1, 14 and 28 of age. However, the antibody titer was induced at day 14 in all inoculated groups and continued to increase at day 28 of age which was significantly different ($p<0.05$) when compared to the control group (Fig., 9 and Appendix 5).

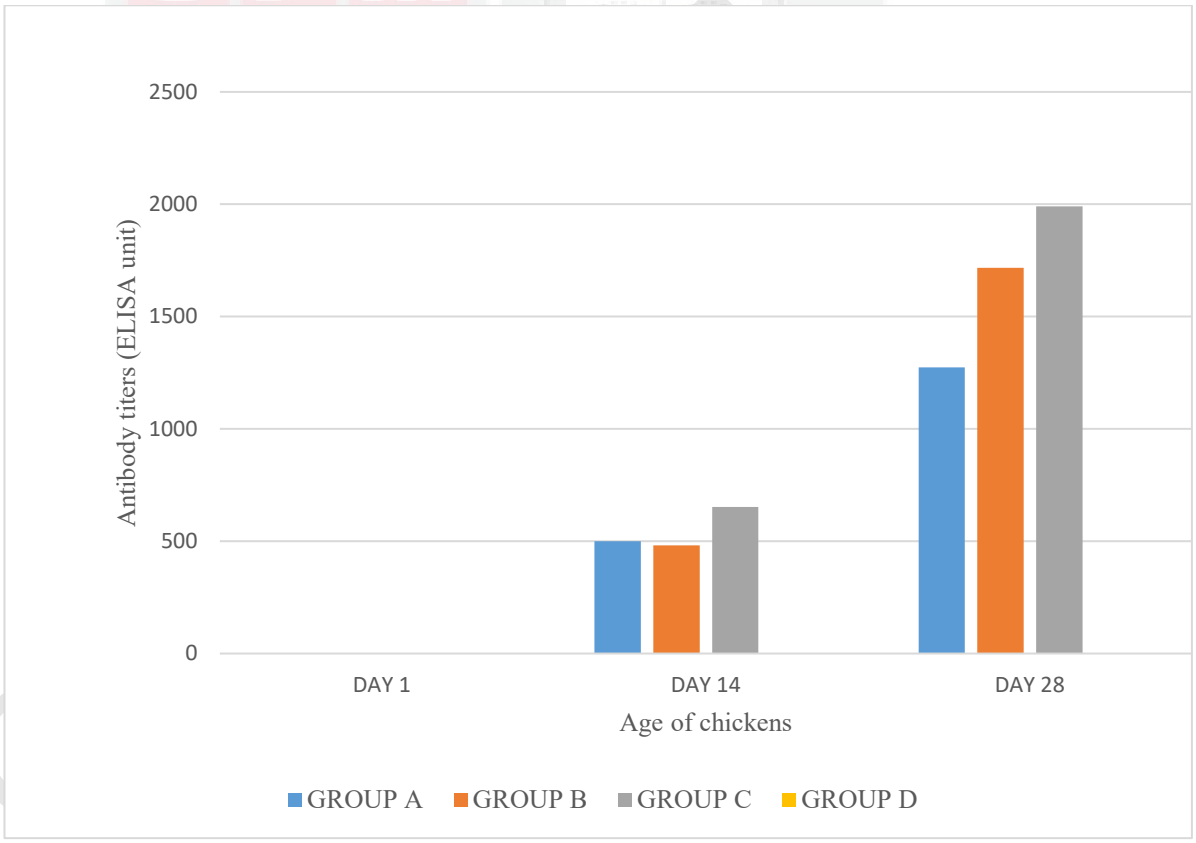


Figure 9: Antibody titer of chickens throughout the trial.

5.0 DISCUSSION

Pathogenicity of the live attenuated FAdV was determined based on four parameters which includes the clinical signs, gross and histological lesions. Whereas the immunogenicity was determined by the FAdV antibody titer following inoculation of attenuated isolates. Based on the current findings, it was confirmed that the FAdV attenuated in CEL cells was low pathogenic and immunogenic in SPF chickens. This finding was in line with study by Mansoor *et al*. (2011) where the attenuated FAdV isolates induce high antibody response with long lasting immunity until 3 weeks post inoculation.

The significant increased of relative liver to body weight in all inoculated groups when compared with control group on day 14 of age might be due to virus replication in liver which was the primary organ affected in IBH outbreak. Then, mild degeneration of cells occurred resulting an immune response.

It was also found that there was no significant different in immunogenicity between all of the FAdV passages. Thus, attenuated isolates were confirmed safe as vaccine candidate without exhibiting clinical signs, mortality and no significant finding in gross and histological lesions. Ojkic and Nagy (2003) mentioned that, the antibody response that was evaluated by ELISA and plaque reduction test depended on the virus dosage and the route of inoculation.

SPF chickens was chosen as subject in this study due to the absence of maternal derived antibody against FAdV and other microorganisms. Besides that, inoculation was done in day-old-chicks to stimulate antibody response within early age to protect

chicks from disease since FAdV infection can occur as early as 7 days old (Hair-Bejo, 2005). Furthermore, the objective of using day-old-chick was to apply for hatchery vaccination. Some of the advantages of hatchery vaccination includes full protection at early age, less work load, less manpower and also reduce stress in chicks due to vaccination.

The numbers of IBH cases were increasing from the first time it was recorded in 2005. Therefore, an effective vaccination strategies against FAdVs infection should be developed in our country to control and prevent IBH outbreaks in commercial broiler chickens (Juliana *et al*., 2014).

6.0 CONCLUSION

The FAdV attenuated in CEL cells is low pathogenic due to absence of abnormal clinical signs, gross and histological lesions and also mortality. It was immunogenic due to the high induction of FAdV antibodies.

7.0 RECOMMENDATIONS

For further studies, it is recommended that the number of samples sizes in the trial should be increased. Besides that, the pathogenicity and immunogenicity of earlier FAdV passages should be tested on. We can also perform a vaccine trial to test the efficacy of vaccine challenged with pathogenic virus. Last but not least, other alternative routes of administration can be considered during the studies.

REFERENCES

Adair, B.M., and Fitzgerald, S.D. (2008). Group I adenovirus infections. In: *Diseases of poultry*, 12th ed. Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan, and D.E. Swayne (Eds). Wiley-Blackwell, Hoboken, NJ. pp. 252–266.

Alemnesh, W., Hair-Bejo, M., Aini, I., and Omar, A.R. (2012). Pathogenicity of fowl adenovirus in specific pathogen free chicken embryos. *Journal of Comparative Pathology*, 146: 223-229.

Alvarado, I.R., Villegas, P., El-Attrache, J., Jensen, E., Rosales, G., Perozo, F., and Purvis, L.B. (2007). Genetic characterization, pathogenicity and protection studies with an avian adenovirus isolate associated with inclusion body hepatitis. *Avian Diseases*, 51: 27-32.

Anjum, A.D. (1990). Experimental transmission of hydropericardium syndrome and protection against it in commercial broiler chickens. *Avian Pathology*, 19: 655-660.

Bancroft, J., and Stevens, A. (1996). *The Haematoxylin and Eosin. Theory and Practice of Histological Techniques.* 4th ed. Ch 6. London, New York & Tokyo: Churchill Livingstone p99-112.

Barua, S., Mondal, B., Sanyal, A., Hemadri, D., Bandyopaghyay, S., and Rai, A. (2005). Sequencing and comparative analysis of hexon gene of fowl adenovirus 4 of Indian origin. *Indian Journal of Biotechnology,* 4(3):367.

Benko M., Harrach B., and Russel W.C., (2000), Family Adenoviridae. In: *Virus Taxonomy, Classification, and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses*, M.H. Van Regenmortel, D.H.L. Bishop, E.B. Carstens, M.K. Estes, S. Lemon et al., (Eds.). Academic Press, San Diego, pp. 227-238.

Benkö, M., Harrach, B., Both, G.W., Russell, W.C., Adair, B.M., Adám, E. *et al.*, (2005), 'Family Adenoviridae', in C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger & L.A. Ball (Eds.), *Virus taxonomy. VIIIth report of the International Committee on Taxonomy of Viruses*, Elsevier, New York, pp. 213–228.

Calnek, B.W., John Barnes, H., Beard, C.W., Reid, W.M. and H.W. Yoder, Jr. (1991). In: *Diseases of Poultry*, 9th. Ed. Ames, Iowa, USA: Iowa State University Press. pp. 552-563.

Cowen, B.S. (1992). Inclusion body hepatitis-anaemia and hydropericadium syndromes: aetiology and control. *Worlds Poultry Science Journal,* 48(3):247-254.

Dahiya, S., Srivastava, R.N., Hess, M., and Gulati, B.R. (2002). Fowl adenovirus serotype 4 associated with outbreaks of infectious hydropericardium in Haryana, India. *Avian Diseases,* 46(1): 230-233.

Erny, K. M., Barr, D.A., and Fahey, K. J. (1991). Molecular characterization of highly virulent fowl adenoviruses associated with outbreaks of inclusion body hepatitis. *Avian Pathology*, 20:597-606.

Gaba, A., Parmar, H., Pal, J.K., and Prajapati, K.S., (2010). Isolation, identification and molecular characterization of inclusion body hepatitis, *Veterinary World,* 3(9): 415-417

Ganesh K., Raghavan R., Gowda R.N.S., Satyanarayana M.L., and Suryanarayana V.V.S., (2002) Purification and characterization of the aetiological agent of hydropericardium hepatitis syndrome from infected liver tissues of broiler chickens. *Tropical Animal Health and Production*, 34: 7-17.

Grimes T.M., King D.J., Kleven S.H., and Fletcher O.J., (1977b) Involvement of type-8 avian adenovirus in the etiology of inclusion body hepatitis. *Avian Diseases*, 21: 26- 38.

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

Helmboldt, C., and Frazier, M. (1963). Avian hepatic inclusion bodies of unknown significance. *Avian Diseases,* 7(4): 446-450.

Juliana, M., Nurulfiza, I., Hair-Bejo, O.M., Rani, A., and Aini, I. (2014). Molecular characterization of fowl adenovirus isolated from inclusion body hepatitis outbreaks in commercial broiler chickens in Malaysia. *Pertanika J. Trop. Agri Sci,* 37:483-497.

Khan, A. A., Sabri, A. N., Mansoor, M. K. and Hussain, I. (2005). Hydropericardium syndrome in Pakistan: a review, *World's Poultry Science Journal*, 8: 647–654

Lal B., Maiti N.K., Oberoi M.S., Sharma S.N., (1991). Cell mediated immune responses of chicks following fowl adenovirus type-1 infection. *Comp. Immunol. Microbiol. Infect,* 14:55–8.

Maartens, L.H., Joubert, H.W., Aitchison, H. and Venter, E.H., (2014). Inclusion body hepatitis associated with an outbreak of fowl adenovirus type 2 and type 8b in broiler flocks in South Africa. *Journal of the South African Veterinary Association,* 85(1): 1-5.

Maiti, N.K. and Sarkar, P., (1997). Humoral immune response of chicks to different clinical isolates of avian adenovirus type-1. *Comparative Immunology Microbiology and Infectious Diseases*, 20: 59–62.

Mansoor, M.K., Hussain, I., Arshad, M., Muhammad, G., Hussaini, M.H., and Mehmood, S. (2009). Molecular characterization of fowl adenovirus serotype 4 (FAdV-4) isolate associated with fowl hydropericardium-hepatitis syndrome in Pakistan. *Pakistan Journal of Zoology*, 41: 269-276.

Mase, M., Chuujou, M., Inoue, T., Nakamura, K., Yamaguchi, S., and Imada, T. (2009). Genetic characterization of fowl adenoviruses isolated from chicken with hydropericardium syndrome in Japan. *Journal of Veterinary Medicines Sciences, 71*: 1455-1458.

McFerran, J. B., and Smyth, J. A. (2000). Avian adenoviruses. *Review of Science and Technology Off. International Epiz*, *19:* 589-601.

McFerran, J., Clarke, J., and Connor, T. (1972). Serological classification of avian adenoviruses. *Archic für die Gesamte Virusforschung,* 39(1-3): 132-139.

Nakamura, K., Mase, M., Yamaguchi, S., Shibahara, T., and Yuasa, N. (1999). Pathologic study of specific-pathogen-free chicks and hens inoculated with adenovirus isolated from hydropericardium syndrome. *Avian Diseases*, *43*: 414-423.

Ojkic, D. and Nagy, E. (2003). Antibody response and virus tissue distribution in chickens inoculated with wild-type and recombinant fowl adenoviruses, *Vaccine,* 22: 42–48.

Ono, M., Okuda, Y., Yazawa, S., Shibata, I., Sato, S., and Okada, K. (2004). Pathogenicty by parental injection of fowl adenovirus isolated from gizzard erosion and resistance to reinfection in adenoviral gizzard erosion in chickens. *Veterinary Pathology*, *41*: 483-489.

Ostertagová, E., and Ostertag, O. (2013). Methodology and Application of Oneway ANOVA. *American Journal of Mechanical Engineering*, *1*(7): 256-261.

Panigrahi, S., Swain, K., Routray, A., Rath, A.P., Sahoo, S., and Ganguly, S., (2016). Inclusion body hepatitis and hydropericardium syndrome in poultry: a brief review, *ARC Journal of Animal and Veterinary Sciences,* 2(4): 12-14.

Rahimi, M., and Haghighi, Z.M.S. (2015). Adenovirus-like inclusion body hepatitis in a flock of broiler chickens in Kermanshah province, Iran. *Veterinary Research Forum*, 6: 95.

Riddell, C. (1987). In: Avian Histopathology, 1st ed. *The American Association of Avian Pathologists*. pp 57-65.

Russell, W. C., (2009). Adenoviruses: update on structure and function, J*ournal of General Virology*, 90: 1–20.

Saifuddin, M., and Wilks, C. (1992). Effects of fowl adenovirus infection on the immune system of chickens. *Journal of Comparative Pathology,* 107(3): 285-294.

Schonewille, E., Jaspers, R., Paul, G. and Hess, M., (2010), Specific-pathogen-free chickens vaccinated with a live FAdV-4 vaccine are fully protected against a severe challenge even in the absence of neutralizing antibodies, *Avian Diseases,* 54: 905–910.

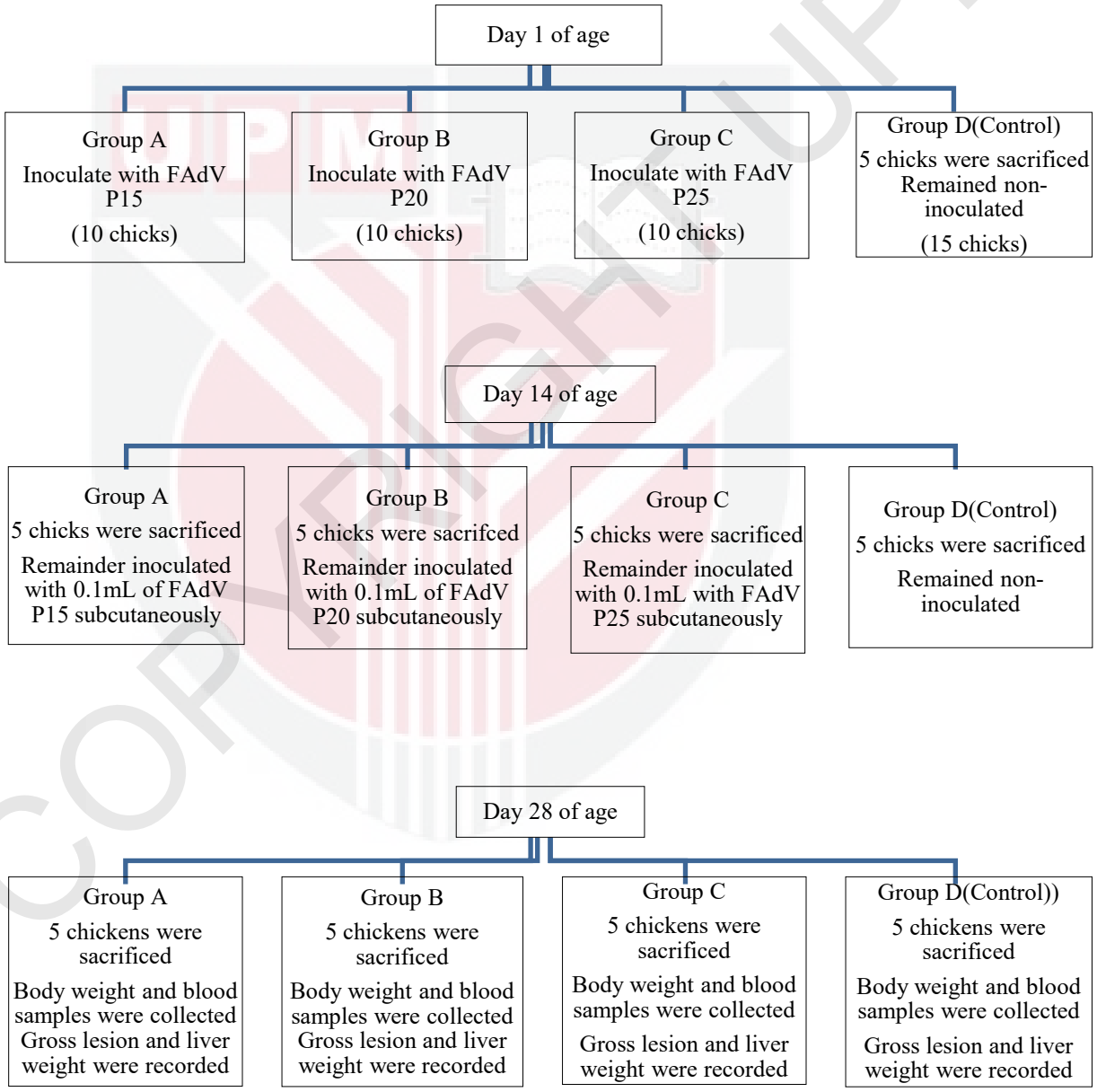
Singh, A., G. S. Grewal, N. K. Maiti, and M. S. Oberoi., (2006). Effect of fowl adenovirus-1 (IBH isolate) on humoral and cellular immune competency of broiler chicks. *Comp. Immunol. Microbiol. Infect. Dis*, 29:315–321.

Toro, H., Gonzalez, C., Cerda, L., Hess, M., Reyes, E. and Geisse, C. (2000).Chicken anemia virus and fowl adenoviruses: association to induce the inclusion body hepatitis/ hydropericardium syndrome. *Avian Diseases*, 51-58.

Zsak, L., and Kisary, L. (1984). Grouping of fowl adenovirus based upon the restriction pattern of DNA generated by BAM HI and Hind III. *Intervirology,* 22:110-114

APPENDIX 1

Table 1: Experimental design for pathogenicity and immunogenicity of FAdV attenuated in CEL cells in SPF chickens.



APPENDIX 2

Table 2: Body weight of the chickens throughout the trial.

Age	Body weight mean (g) ± SEM			
	Group A	Group B	Group C	Group D
1	40.6±0.51			
14	123.5±5.44 [a]	124.0±6.45 [a]	107.5±12.81 [a]	135.0±10.67 [a]
28	294.0±21.00 [a]	317.4±23.48 [a]	274.6±17.28 [a]	323.2±9.70 [a]

Each value is the body weight mean±SEM of 5 chickens from each group. [a,b] Different superscripts indicate significant difference ($p<0.05$).

APPENDIX 3

Table 3: Liver weight of chickens throughout the trial.

Age	Liver weight mean (g) ± SEM			
	Group A	Group B	Group C	Group D
1	1.4±0.10			
14	5.4±0.09 [b]	5.2±0.07 [a,b]	4.3±0.53 [a]	4.6±0.18 [a,b]
28	8.9±0.64 [a]	9.2±0.63 [a]	9.1±0.48 [a]	11.0±0.47 [a]

Each value is the liver weight mean±SEM of 5 chickens from each group. [a,b] Different superscripts indicate significant difference ($p<0.05$).

APPENDIX 4

Table 4: Liver to body weight ratio throughout the trial.

Age	Liver weight:Body weight (mean ± SEM) ($x10^{-3}$)			
	Group A	Group B	Group C	Group D
1	35.6±2.7			
14	43.7±1.4 [b]	42.1±2.4 [a,b]	39.7±1.2 [a,b]	34.9±1.9 [a]
28	30.5±1.4 [a,b]	29.2±0.7 [a]	33.3±1.2 [a,b]	34.0±0.5 [b]

Each value is the relative liver to body weight mean±SEM of 5 chickens from each group. [a,b] Different superscripts indicate significant difference ($p<0.05$).

APPENDIX 5

Table 5: Antibody titer of chickens throughout the trial.

Age	Antibody titer mean ± SEM			
	Group A	Group B	Group C	Group D
1	Not detected			
14	499.8±255.70 [a]	480.8±344.11[a]	652.5±411.20 [a]	Not detected
28	1247.4±373.22 [a]	1716.4±635.18 [a]	1991.0±488.54 [a]	Not detected

Each value is the antibody titer mean±SEM of 5 chickens from each group. [a,b] Different superscripts indicate significant difference ($p<0.05$).