UNIVERSITI PUTRA MALAYSIA

METABOLIC DISORDER AND VIRUS DETECTION IN ORGANS OF CHICKEN FOLLOWING INFECTION WITH FOWL ADENOVIRUS SEROTYPE 8B

MUHAMMAD AIDIL HAIKAL BIN MOHD DZILAN

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203982

Dissertation submitted in partial fulfilment of the requirement for the course VPD4999- Final Year Project in the Department of Veterinary Clinical Studies Universiti Putra Malaysia

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CERTIFICATION

It is hereby certified that we have read this project paper entitle " Metabolic Disorder And Virus Detection In Organs Of Chicken Following Infection With Fowl Adenovirus Serotype 8b" by **Muhammad Aidil Haikal bin Mohd Dzilan** and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4901-Project.

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ABBREVIATIONS

FAdV	Fowl Adenovirus
FAdV-8b	Fowl Adenovirus serotype 8b
IBH	Inclusion Body Hepatitis
AGE	Adenoviral Gizzard Erosion
HHS	Hepatitis Hydropericardium Syndrome
ELISA	Enzyme-linked Immunosorbent Assay
PCR	Polymerase Chain Reaction
AST	Aspartate aminotransferase
GGT	Gamma-glutamyltransferase
CK	Creatine kinase

ABSTRAK

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

GANGGUAN METABOLIK DAN PENGESANAN VIRUS DALAM ORGAN AYAM YANG DIJANGKITI DENGAN SEROTIP 8B ADENOVIRUS UNGGAS

Oleh

Muhammad Aidil Haikal bin Mohd Dzilan

2023

Pengerusi: Dr. Norfitriah Mohamed Sohaimi

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Dr. Azalea Othman

Adenovirus unggas (FAdV) ialah virus DNA untai dua yang tidak bersampul dan ia bertanggungjawab terhadap pelbagai penyakit dalam ayam termasuk *inclusion body hepatitis* (IBH), sindrom hepatitis hidroperikardium (HHS), dan hakisan hempedu adenoviral (AGE). Matlamat kajian adalah untuk menentukan parameter metabolik dan pengesanan virus dalam organ ayam berikutan jangkitan FAdV serotype 8b. Lima belas ekor anak ayam bebas patogen khusus (SPF) berumur sehari dibahagikan kepada dua kumpulan iaitu, kumpulan yang dijangkiti dan kumpulan kawalan. Enam anak ayam telah ditetapkan dalam kumpulan yang dijangkiti dan sembilan anak ayam dalam kumpulan kawalan. Semua anak ayam dalam kumpulan yang dijangkiti

telah diinokulasi dengan 1ml isolat FAdV, UPM1901, pada titer virus $10^{7.1}TCID_{50}$/ml melalui laluan oral pada umur 7 hari. Semua anak ayam dalam kawalan kekal tanpa inokulasi dalam kajian ini. Semua anak ayam dipantau untuk sebarang tanda klinikal yang berkaitan dengan IBH sepanjang percubaan. Semasa pensampelan, berat badan, berat hati dan berat bursa Fabricius diukur termasuk pengumpulan darah untuk analisis biokimia serum pada hari 0 pasca inokulasi (pi) untuk kumpulan kawalan dan hari ke-3 dan ke-7pi dalam kedua-dua kumpulan. Sampel hati, timus, tonsil cecal, ginjal, bursa Fabricius, limpa, dan sumsum tulang telah dikumpulkan untuk pemeriksaan histologi dan analisis *polymerase chain reaction* (PCR). Kajian ini menunjukkan bahawa ayam yang dijangkiti FAdV menjadi murung, bulu yang kusut dan hilang selera makan pada hari ke-6pi. Selepas nekropsi, bengkak, pendarahan dan nekrosis hati diperhatikan dalam ayam dari kumpulan yang dijangkiti FAdV pada hari ke-3pi. Berat hati ayam bagi kumpulan yang dijangkiti telah meningkat dengan ketara ($p<0.05$) pada hari ke-3pi (5.3 ± 0.1) dan hari ke-7pi (5.6 ± 0.6) berbanding kumpulan kawalan. Di samping itu, nisbah hati kepada berat badan juga jauh lebih tinggi dengan signifikan ($p<0.05$) pada hari ke-3 dan ke-7pi daripada kumpulan kawalan akibat pembesaran hati. Banyak *basophilic intranuclear inclusion bodies* (INIB) dilihat di dalam hepatosit pada hari ke-3 dan ke-7pi kumpulan yang dijangkiti yang merupakan indikator zarah virus wujud di dalam organ hati. Keputusan biokimia serum menunjukkan enzim hati untuk aspartate-aminotransferase (AST) dan paras gamma-glutamyl transferase (GGT) meningkat dalam kumpulan ayam yang dijangkiti FAdV pada hari ke-3 dan 7pi tetapi paras *creatine kinase* (CK) lebih rendah daripada kumpulan kawalan mungkin disebabkan oleh kecederaan hati yang disebabkan oleh jangkitan FAdV. FAdV dikesan oleh PCR dalam organ hati, ginjal dan limfoid pada hari ke-3 dan ke-7pi dalam kumpulan yang dijangkiti. Ini mengesahkan bahawa FAdV serotip 8b isolat UPM1901, menyebabkan gangguan metabolik dalam ayam SPF berikutan inokulasi pada

umur 7 hari dengan bukti distribusi virus dalam pelbagai organ.

Kata kunci: Adenovirus unggas (FAdV), patogenik, metabolik, enzim hati, pengesanan virus.

ABSTRACT

An abstract of the project presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4901- Project.

METABOLIC DISORDER AND VIRUS DETECTION IN ORGANS OF CHICKEN FOLLOWING INFECTION WITH FOWL ADENOVIRUS SEROTYPE 8B

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2023

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Fowl adenoviruses (FAdVs) are non-enveloped double-stranded DNA viruses and they are responsible for a variety of diseases in chickens including inclusion body hepatitis (IBH), hydropericardium hepatitis syndrome (HHS), and adenoviral gizzard erosion (AGE). The aim of study is to determine the metabolic parameters and virus detection in organs of chickens following infection with FAdV serotype 8b. Fifteen, day-old specific pathogen free (SPF) chicks were divided into two groups namely, infected group and control group. Six chicks were assigned in infected group and nine chicks in the control group. All chicks in the infected group were inoculated with 1ml FAdV isolate, UPM1901, at virus titer $10^{7.1}TCID_{50}$/ml via oral route at day 7 of age. All chicks in the control remained uninoculated in this study. All chicks were monitored

for any clinical signs associated with IBH throughout the trial. During sampling, body weight, liver and bursa weight were measured including blood collection for serum biochemistry analysis at day 0 post-inoculation (pi) for control group and at day 3 and 7pi in both groups. Samples of liver, thymus, cecal tonsil, kidney, bursa of Fabricius, spleen, and bone marrow were collected for histological examination and polymerase chain reaction (PCR) analysis. It was demonstrated that infected chickens with FAdV were depressed, ruffled feathers and inappetence at day 6pi. Upon necropsy, swollen, haemorrhage and necrosis of liver was observed in chicken from infected group with FAdV at day 3pi. Liver weight of chicken for infected group was increased significantly (p<0.05) at day 3pi (5.3 ± 0.1) and day 7pi (5.6 ± 0.6) compared to the control group. In addition, liver to body weight ratio was also significantly higher (p<0.05) at day 3 and 7pi than the control group due to enlarged liver. Numerous basophilic intranuclear inclusion body (INIB) was observed in the hepatocytes at day 3 and 7pi of the infected group which is an indicator of viral particles exists in the liver organ. Serum biochemistry results showed liver enzymes for aspartate-aminotransferase (AST) and gamma-glutamyl transferase (GGT) level were elevated in chickens from FAdV infected group at day 3 and 7pi but lower creatine kinase (CK) level than control group probably due to liver injury caused by FAdV infection. FAdV was detected by PCR in liver, kidney and lymphoid organs at day 3 and 7pi in group A. It was confirmed that the FAdV isolate serotype 8b, UPM1901, induces metabolic disturbances in SPF chicken following inoculation at 7 days old with evidence of virus distribution in various organs.

Keywords: Fowl adenovirus (FAdV), pathogenic, metabolic, liver enzymes, virus detection.

CHAPTER 1

INTRODUCTION

1.1 Background

Fowl adenoviruses (FAdVs) are non-enveloped double-stranded DNA viruses, which belong to the genus *Aviadenoviridae*, under the *Adenoviridae* family and they can be spread by vertically and horizontally (Chandra *et al*., 2000). FAdVs can be classified into five different species which are FAdV-A to FAdV-E and have 12 serotypes which are FAdV-1 to -8a and 8b to -11 (Fauquet *et al*., 2008). Among these, FAdV-E includes FAdV-6, FAdV-7, FAdV-8a and FAdV-8b. Hexon, penton and fiber are the main protein structure of the FAdVs capsid. The hexon and fiber genes are highly prone to mutation. The infectivity markers for FAdV infection seem to be associated with the L1 loop of hexon and the knob of fiber genes (Meulemans *et al*., 2001; Sohaimi *et al*., 2021).

FAdVs are common infectious agents in fowl worldwide. Typical FAdV infection in chickens were inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS) and adenoviral gizzard erosion (AGE) caused by pathogenic FAdV strains and it led to economic losses on a global scale (Mazaheri *et al*., 1998; Balamurugan and Kataria, 2004; El-Shall *et al*., 2022). For cases of IBH, strains predominantly belonging to species FAdV-D and FAdV-E have been isolated in many nations (Kajan *et al.,* 2019).

Virulence FAdV caused high mortality and severe lesions in multiple organs with immunosuppressive effect in infected SPF chickens (Niu *et al*., 2017). In Malaysia, IBH outbreak was first reported in Perak state in 2005 and subsequently in other states as reported by previous works (Norina *et al*., 2016; Sohaimi *et al*., 2022). FAdV serotype 8b was identified as the primary

pathogen of IBH and highly pathogenic in SPF chickens (Sohaimi *et al*., 2019).

Gross and histopathological lesion of affected chickens indicated that liver lesions were predominant, particularly in IBH cases. The liver exhibited enlargement, pale, and friable, with histopathological findings revealing multifocal inflammation and necrosis (Tsiouris *et al*., 2022). Additionally, characteristic large basophilic intranuclear inclusion bodies were observed in hepatocytes (Tsiouris *et al*., 2022). In certain instances, there was observed diffuse vacuolization of lymphoid follicles, alongside pronounced lymphocytolysis in medullary lymphocytes, as reported in previous studies (Ren *et al*., 2019).

Phylogenetic analyses of partial hexon gene sequences are an adequate and quick method for differentiation and genotyping of FAdVs (Morshed *et al.,* 2017). Polymerase chain reaction (PCR) with primer sequences based on the hexon gene is useful for virus detection (Wang *et al*., 2017). Hexon, being the major gene of the adenovirus, is recognized for harboring the neutralizing epitope (Liu *et al.,* 2016). Consequently, sequencing the hexon gene is a commonly employed method for FAdVs serotyping. Liver is the major tropism for FAdV replication and caused severe damages as it was characterized by a high level of coxsackievirus-adenovirus receptor (CAR) expression and integrin receptors that bind to adenovirus fibers, serves as a trigger for infection (Wang *et al*., 2023).

1.2 Justification

In recent years, the poultry industry has faced significant economic losses primarily due to high mortality rates and reduced production on commercial farms, all attributed to IBH triggered by FAdV serotype 8b. Analysis on metabolic disturbances caused by FAdV serotype 8b infection has remained unknown in chicken. Although FAdV is highly pathogenic in SPF chicken, yet, study on

the complete blood and serum biochemistry profiles is scanty and limited. It is important to highlight the effect of the FAdV infection in order to strategize the effective control and treatment against the disease outbreak in a local poultry farm.

1.3 Hypothesis

Null hypothesis: Blood, serum biochemistry profiles and histological finding are normal in both control and infected chickens with undetectable virus following inoculation with FAdV serotype 8b isolate UPM1901.

Alternate hypothesis: Blood and serum biochemistry profiles are abnormal in the infected chickens with presence of histological lesions in the liver and detectable virus following inoculation with FAdV serotype 8b isolate UPM1901.

1.4 Objectives

The objective of this study are:

1. To evaluate the metabolic parameters in chickens following inoculation with FAdV serotype 8b isolate UPM1901.
2. To detect presence of FAdV in liver and lymphoid organs by molecular method following inoculation with FAdV serotype 8b isolate UPM1901.

CHAPTER 2

LITERATURE REVIEW

2.1 Aetiology and Classification of Fowl Adenovirus

Fowl adenoviruses (FAdVs) belongs to *Adenoviridae* family under Aviadenovirus genus. FAdV has been identified as aetiological agent of several diseases, such as inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), gizzard erosions and proventriculitis. These viruses are categorized into five species based on their molecular structure and further classified into 12 serotypes through cross-neutralization tests. Based on International Committee on Taxonomy of Viruses (ICTV) 9th report (2011), five FAdV species and their corresponding serotypes as follows: FAdV A (FAdV-1), FAdV B (FAdV-5), FAdV C (FAdV-4 and -10), FAdV D (FAdV-2, -3, -9, and -11), and FAdV E (FAdV-6, -7, -8a, and -8b) (ICTV, 2011). Among the various diseases caused by FAdVs in poultry, IBH and HHS are particularly significant and have been documented in various regions worldwide. All 12 serotypes have been associated with IBH, while serotype 4 has been implicated in HHS (Xie *et al*., 2022; Mittal *et al*., 2013).

2.2 Inclusion Body Hepatitis in Chicken

According to Hair-Bejo (2005), IBH is characterized by a rapid onset of mortality that peaks around 3-4 days after infection, ending on the fifth day, although there are instances where the infection may persist for 2-3 weeks. IBH typically manifests in broiler chickens aged 3 to 7 weeks, although cases have been documented in birds as young as 7 days and as old as 20 weeks (Rahimi and Minoosh Siavosh Haghighi, 2015). Natural outbreaks are characterized by a sudden onset of mortality ranging from 2 to 40 percent in chickens. High mortality is observed particularly in birds younger than 3-week-old, and depending on the virus's pathogenicity, the chicks' immune status,

and concurrent secondary infections, mortality rates of up to 80% have been reported. Mortality usually peaks within three to four days and subsides within 9-14 days. Clinically, affected birds exhibit lethargy, huddling, ruffled feathers, and a lack of appetite (Hafez *et al*., 2011). In most cases, the main lesions are found in the liver as the gross lesions associated with IBH include an enlarged, pale, and friable liver, sometimes with necrotic foci. Ecchymotic haemorrhages may also be present in the liver and, to a lesser extent, in leg and breast muscles (El-Tholoth *et al*., 2019).

2.3 Diagnosis of Fowl Adenovirus

A tentative diagnosis typically relies on the observation of a spiking mortality pattern and distinctive gross lesions. The confirmation of the diagnosis involves a microscopic examination of the affected tissues, specifically identifying characteristic lesions, such as intranuclear inclusion bodies, or performing PCR (Schachner *et al*., 2017). From a macroscopic perspective, birds impacted by the condition typically exhibit livers that are pale yellow, friable, and swollen. Additionally, petechial and/or ecchymotic haemorrhage may be evident in both the liver and muscles (El-Tholoth *et al*., 2019). Degenerated hepatocytes often reveal two types of intranuclear inclusion bodies (INIB) during microscopic examination. These can be either large and round or irregularly shaped, featuring a distinct pale halo with eosinophilic or basophilic inclusions that occupy the nucleus (Safwat *et al*., 2022).

The histopathological changes observed in experimental birds infected with fowl adenovirus revealed vacuolar degeneration in all hepatic tissues and the presence of intranuclear inclusion bodies after further confirmed through electron microscopic examination (Chen *et al*., 2019). Various serological techniques have been conducted for the diagnosis of FAdV infections in poultry. These encompass the agar gel immunodiffusion (AGID) test, agar gel precipitation test, indirect hemagglutination, viral neutralisation test (VNT), indirect immunofluorescence assay, and

several modified enzyme-linked immunosorbent assay (ELISA) approaches. Initially, using prepared hyperimmune sera, agar gel immunodiffusion (Li *et al*., 2017), agar gel precipitation test (Kumar *et al*., 2003), and indirect immunofluorescence assay (Kumar *et al*., 2003) successfully detected FAdV in liver homogenate extracts or different tissues of affected birds. Indirect hemagglutination was used to measure antibody titer and seroprevalence post-vaccination and to detect HHS agent antibodies in infected chickens (Li *et al*., 2017). However, the VNT method is more sensitive and accurate, but it also has disadvantages due to its expense and time-consuming nature (Zhang *et al*., 2022) and should be used judiciously.

Recent advancements in FAdV serological techniques have primarily focused on modified ELISA methods. In earlier versions, an indirect ELISA with whole viruses as coating antigens was utilized to detect antibodies against FAdV in tissue samples from chickens experiencing natural and experimental infections (Hao *et al*., 2020; Kumar *et al*., 2003). Recently, successful expression and purification of non-structural proteins (100 K, 33 K) and structural proteins (hexon, fibre-2) in a prokaryotic expression system have led to the development of sensitive, specific, and accurate indirect ELISA methods based on recombinant proteins. Importantly, these methods are easier to standardise, making them suitable for large-scale applications (Junnu *et al*., 2014). PCR serves as the main method for FAdV detection (Hou *et al*., 2022). The avian adenovirus PCR assay is esteemed for its high sensitivity, simplicity, selectivity, and rapidity (Hess *et al.,* 2000; Asthana *et al*., 2013). The hexon gene of adenoviruses is composed of conserved pedestal regions (P1, P2) and variable loops (L1–L4) (Sohaimi and Bejo, 2021) and represents the primary target gene for published PCR techniques in avian adenovirus detection. The utilization of PCR with two primer pairs, H1/H2 and H3/H4, which bind to the conserved region, in conjunction with restriction enzyme analysis (REA), facilitates the detection and differentiation of all 12 FAdV reference

strains (Raue and Hess, 1998; Meulemans *et al*., 2001; Singh *et al*., 2002; Meulemans *et al*., 2004). Presently, the PCR product of the hexon gene is directly sequenced, and the resulting information allows for the identification of the group and type of avian adenovirus (Li *et al*., 2017). Additionally, fiber genes are employed for avian adenovirus detection due to their encoding of type-specific neutralizing, type-specific non-neutralizing, and subgenus-specific neutralizing epitopes.

Virus isolation and identification routinely used virus-targeted samples such as liver, faeces, kidney, gizzard and pharynx. In cases of IBH, liver, kidney, or hepatocellular cancer cell lines from chick embryos are infected with a 10% suspension of the sample material (El-Shall *et al*., 2022). While chicken cells can be utilized, it is more advisable to employ cells from the same avian species when attempting to isolate adenoviruses from other avian species (Hess *et al*., 2000). The successful isolation of most avian adenoviruses from embryonated eggs is challenging, although certain FAdV isolates have demonstrated the ability to infect the yolk sac. The identification of an adenovirus isolate can be confirmed through electron microscopy (Bodewes *et al*., 2013). Immunocytochemistry, a technique for detecting adenoviruses, involves staining infected cells with FAdV antiserum labelled with a fluorescent dye. To determine the serotype of an isolated virus, virus neutralization testing is essential, utilizing the isolate against common reference antisera for each known serotype (Hess *et al*., 2000).

2.4 Worldwide Distribution of Fowl Adenovirus causing Inclusion Body Hepatitis

The first case of FAdV causing IBH was reported in 1963 in the USA (Cizmecigil *et al*., 2020). Since then, the virus was spread globally caused by various serotypes in different countries. The primary agent of IBH consist of serotype 4, 8b, 9 and 11 (Sohaimi and Clifford, 2021). Among European countries, FAdV serotype 2, 8a, 8b, and 11 were identified as the causative agents of

IBH in both broiler and breeder flocks in Spain from 2011 to 2013 (Oliver *et al*., 2016). Instances of IBH have been documented in Slovenia, Poland, Hungary, Greece, Belgium, as well as in Germany, France, and Austria with a predominant presence of FAdV isolates falling under species D and E, consistent with the global trend (Cizmecigil *et al*., 2020). From 2001 onwards, there has been a rise in IBH outbreaks linked to FAdVs in Canada, resulting in significant economic losses for the poultry industry (Ojkić *et al*., 2008). Several studies have demonstrated less to severe degrees of clinical signs and mortality in chickens of different ages, ranging from 1 day to 3 weeks old, when exposed to FAdV serotypes 8b and 11 (Islam *et al*., 2023).

2.5 Metabolic Disturbances Caused by Fowl Adenovirus

Specific clinical chemistry analytes, indicative of cellular integrity and physiological function in the liver and pancreas, were utilized as biomarkers to assess strain pathogenicity and pathogenesis of IBH (Matos *et al*., 2016). This approach revealed disruptions in various enzymatic systems and alterations in metabolite concentrations. Notably, acute changes in plasmatic lipase levels, along with severe histopathological lesions, pointed to the pancreas as a significant target organ in IBH pathogenesis. In subsequent experiments inducing IBH, day-old SPF broilers that were orally infected displayed clinical signs of hypoglycemia, severe hepatitis, and pancreatitis (Matos *et al*., 2016).

Earlier observations by Goodwin *et al*. (1993) described a flock with spiking mortality, where broilers experiencing hypoglycemia exhibited intranuclear inclusion bodies in the liver, pancreas, and small intestine. This finding potentially elucidates why IBH outbreaks are predominantly reported in broilers, given their physiological differences from layers. Intriguingly, broilers suffering from hypoglycemia, metabolic acidosis, and hypocalcemia during an IBH outbreak responded well to treatment with glucose, sodium bicarbonate, and calcium, underscoring the

metabolic aspect of IBH pathogenesis (Matos *et al*., 2016).

From previous researches, liver injury resulting from FAdV Serotype 4 (FAdV-4) infection exhibits distinct features, including small multifocal necrosis, mononuclear cell infiltration, vacuolar degeneration, and the presence of basophilic intranuclear inclusion bodies within hepatocytes (Niu Y *et al*., 2017). In alignment with findings reported by (Niu *et al*., 2018), FAdV-4-infected chickens displayed decreases in concentrations of blood albumin (ALB) and glucose (GLU), along with increases in plasma enzyme activity. These observations are likely directly linked to the hepatic damage induced by FAdV-4 infection. The hepatic steatosis associated with FAdV-4 infection is characterised by the cytoplasmic accumulation of lipid droplets. There was connection between FAdV-4-induced hepatocyte damage and hepatic steatosis, substantiated by the sustained accumulation of intracellular oil droplets, primarily triglycerides, which serves as a reliable indicator of hepatic steatosis (Yan *et al*., 2021).

2.6 Management Strategies to Mitigate Fowl Adenovirus Diseases

The prevention of FAdV infection primarily relies on implementing biosecurity measures. Stringent management practices, thorough cleaning and disinfection of premises and equipment, restricting entry and/or ensuring personal protection for visitors and vaccination crews entering poultry houses, all contribute significantly to preventing IBH and hepatitis-hydropericardium syndrome (HHS) (El-Shall *et al*., 2022).

Successful prevention of gizzard erosion caused by FAdV-1 infection was achieved through the application of a combination of glutaraldehyde and calcium hydroxide liquid under ambient temperature (21°C) during downtime both inside and outside the house (Inoue *et al.,* 2020). However, it's worth noting that the susceptibility of human adenovirus to disinfectants varies

depending on the strain, and some strains show reduced susceptibility to disinfectants such as liposomal povidone-iodine, peracetic acid, and formaldehyde (Sauerbrei *et al*., 2004). Eradicating avian adenoviruses once they have infected chicken farms poses a significant challenge, and these viruses are considered latent across the country (Yamaguchi *et al*., 2022).

Based on Yamaguchi *et al*., (2022) work, isolated and identified serotype 1 from FAdV species A causing gizzard erosion, as well as serotype 5 from FAdV species B and serotype 8b from FAdV species E from apparently healthy broiler and layer breeder chickens aged 1–20 weeks in Japan. Similarly, Meng *et al*., (2018) isolated FAdV serotype 7 from parental and offspring generations of chickens, along with co-infections with avian reticuloendotheliosis virus (REV), avian leukosis virus (ALV), and chicken infectious anemia virus (CIAV). Consequently, effective FAdV control should commence at the primary breeder level, involving optimal disinfection and vaccination as parallel strategies to prevent infection and protect against vertical transmission. However, addressing horizontal spread is equally important, requiring concerted efforts to maintain a commercial flock free of FAdV infection. Additionally, controlling and/or eliminating immunosuppressive diseases like infectious bursal disease virus (IBDV) and chicken infectious anaemia virus (CIAV) is critical in reducing FAdV disease, as these conditions enhance FAdV pathogenicity (El-Shall *et al*., 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1 Virus Isolate

FAdV isolate namely, UPM1901 isolate was obtained from an outbreak in Johore in 2019 from 25-day-old commercial broiler chickens with a history of 1.22% mortality. Upon necropsy, the liver was swollen, necrotized and hemorrhages in dead chickens. The liver was processed and passaged in SPF chicken embryonated eggs and determined for virus titration based on Reed and Muench (1939) method. The isolate was confirmed FAdV serotype 8b based on molecular characterization (Sohaimi *et al*., 2022).

3.2 Source of chicks

Fifteen (15) specific pathogenic free (SPF) chicken embryonated eggs (CEE) were purchased from Malaysia Vaccine and Pharmaceutical Sdn. Bhd. All CEEs were kept in the hatcher until hatch and all day-old chicks were transferred into Animal House in the Biologic Laboratory in Faculty of Veterinary Medicine, Universiti Putra Malaysia.

3.3 Experimental Design in the Chicken Trial

The study was conducted under IACUC reference number: UPM/IACUC/AUP-U015/2023. Fifteen (15) day-old chicks were divided into 2 major groups namely, FAdV infected group and control group. Six (6) chicks was assigned in FAdV infected group and nine (9) chicks in control group. All chicks in the infected group were inoculated with 1ml FAdV isolate, UPM1901, at virus titer $10^{7.1}TCID_{50}$/ml via oral route at 7 days old. While, all chicks in the control group remained uninoculated and used as a control group in this study. All chickens were monitored throughout the trial and both feed and water were given *ad libitum*. At day 0 post-inoculation (pi), body weight

was recorded prior necropsy and whole blood and serum was collected for complete blood count (CBC) and serum biochemistry analysis from three chicks in the control group prior sacrificed by cervical dislocation. Samples of liver, thymus, caecal tonsil, kidney, bursa of Fabricius, spleen, and bone marrow were collected from the chicks for histological examination and virus detection via PCR analysis (Sohaimi *et al*., 2021). In addition, liver and bursa of Fabricius were weighed for all chickens following necropsy. Sampling was performed subsequently in both groups at days 3 and 7pi (Appendix 1, Table 1).

3.4 Serum Biochemistry

Blood was collected from all chicks for analysis of complete blood count (CBC). Serum sample was further analyzed for serum biochemistry parameter for liver enzyme such as aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and creatine kinase (CK) (Lippi *et al*., 2011).

3.5 Histological Examination

Sample of liver was harvested from the chicken and fixed in 10% buffer formalin for histological examination (Matos *et al.,* 2016). Upon using light microscope, the slide glass slide was placed onto the stage. Observation on slide was started with lowest power objective lens with 100x. The coarse and fine focus knob were adjusted until can focus the cells clearly. Then, the power of objective lens was adjusted to 200x and 400x to see the cells in more detail. The slide also can be viewed on the screen of desktop computer as it was connected with microscope in order to capture the image of histological slide. All samples were analyzed and described based on severity of the lesions (Sohaimi *et al*., 2021).

3.6 Molecular Detection by Conventional Polymerase Chain Reaction

All the tissue samples from chickens were extracted using a DNA extraction kit (Kylt® RNA/DNA purification) according to protocol by the manufacturer. The eluted DNA was measured for concentration and DNA purity by using biophotometer (Eppendorf, Germany). Extracted DNA sample was used as a template for amplifying the hexon gene using MyTaq™ HS Mix (Bioline, UK) and following the standard MyTaq HS Mix Protocol with using the hexon gene primer namely Hexon A (Forward) and Hexon B (Reverse) (Sohaimi *et al.,* 2022). The PCR products were separated in a 1.5% agarose gel electrophoresis using RedSafe™ Nucleic Acid Staining solution (iNtRON, Korea) and 100bp DNA Marker (GeneDirex, USA). Electrophoresis was conducted at 110 volts for 25 minutes prior visualisation of DNA fragment band under U.V. transillumination (Günes *et al*., 2012; Sohaimi *et al*., 2021).

3.7 Statistical Analysis

Mean body weight, liver weight, bursa of Fabricius weight, liver-to-body weight ratio and serum chemistry data were analyzed using statistical data analysis software using SPSS Version 27. The data was compared between FAdV infected group and control chickens using independent T tests based on the day of sampling and comparison within these two groups using one-way analysis of variance (ANOVA). The significant difference was measured at alpha $p < 0.05$ value between groups (Zhou *et al*., 2023).

CHAPTER 4

RESULT AND DISCUSSION

4.1 RESULTS

4.1.1 Clinical Signs

FAdV infected chicks showed clinical signs associated with IBH such as lethargic, depression, ruffled feathers, inappetence and watery greenish dropping starting from day 2 post-inoculation (pi). Whereas there is no clinical sign was observed for control group chickens.

Figure 1: Chicken in FAdV infected group showed clinical signs of depression and ruffled feathers at day 6 post-inoculation (pi).

4.1.2 Body Weight

Mean body weight for FAdV infected group was 83.6g ± 4.6 which increased significantly (p<0.05) at day 3pi compared to the control group was 58g ± 6.7 (Figure 2). At day 7pi, there is no significant differences (p>0.05) in body weight for both groups. This can be clearly described in Appendix 2, Table 2.

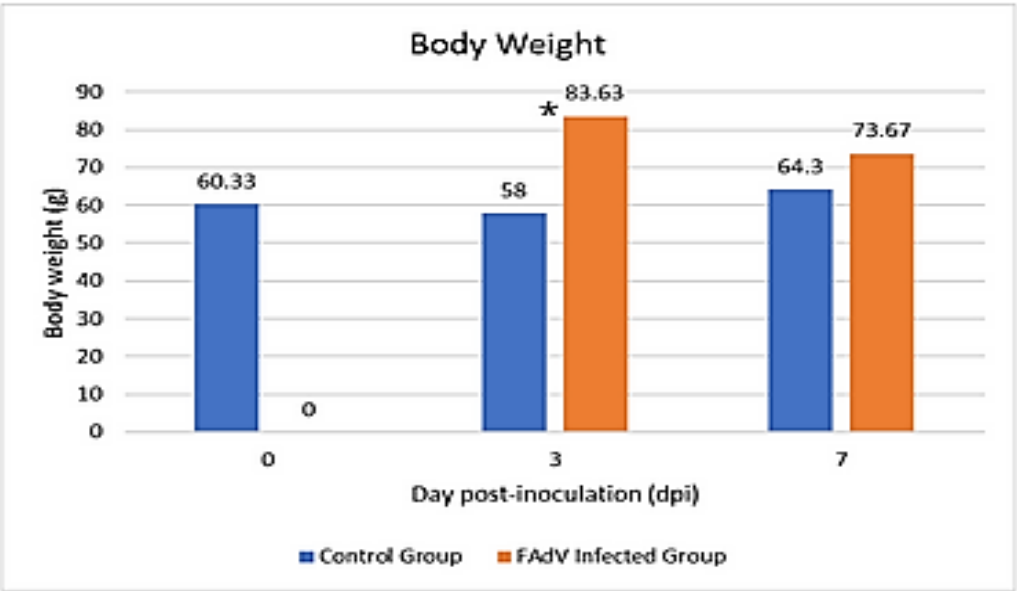

Figure 2: Mean of body weight of the chickens between control and FAdV infected groups from day 0 post-inoculation (pi) until day 7pi. *asterisk indicate significant different between group.

4.1.3 Gross Lesions

At day 3pi for the control group, the liver was normal with brownish color and glistening appearance. Whereas, the liver in infected group showed swollen, enlarged, yellowish discoloration, congested and friable. There was no abnormal finding to other tissues at day 3pi. At day 7pi for the control group, the liver was normal as shown in Figure 3a. On the other hand, the liver for infected group was swollen, petechial hemorrhagic, friable and necrosis as demonstrated in Figure 3b. Moreover, the gross lesion was observed in the kidney such as swollen, pale with urate deposition.

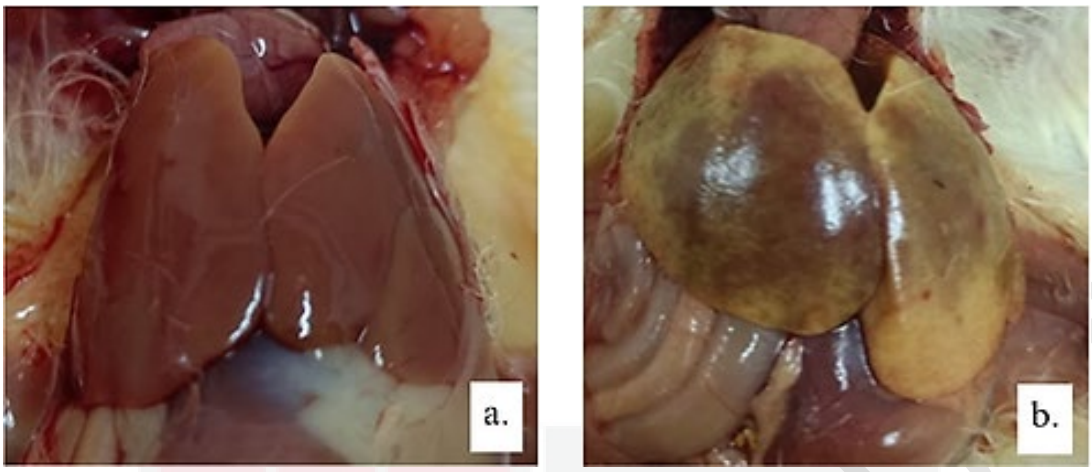

Figure 3: Necropsy finding of liver in chickens between control and FAdV infected groups at day 7 post-inoculation. **(a):** Normal liver in the control group with brownish color and glistening appearance, **(b):** Liver of FAdV infected chicken with swollen, petechial hemorrhagic, friable and necrosis after inoculated with FAdV-8b isolate UPM1901.

4.1.4 Liver Weight

Mean liver weight of chicken for infected group was increased significantly (p<0.05) at day 3pi which was 5.3g ± 0.1 and day 7pi which was 5.6g ± 0.6 as compared to the control group (Figure 4). This can be clearly described in Appendix 3, Table 3.

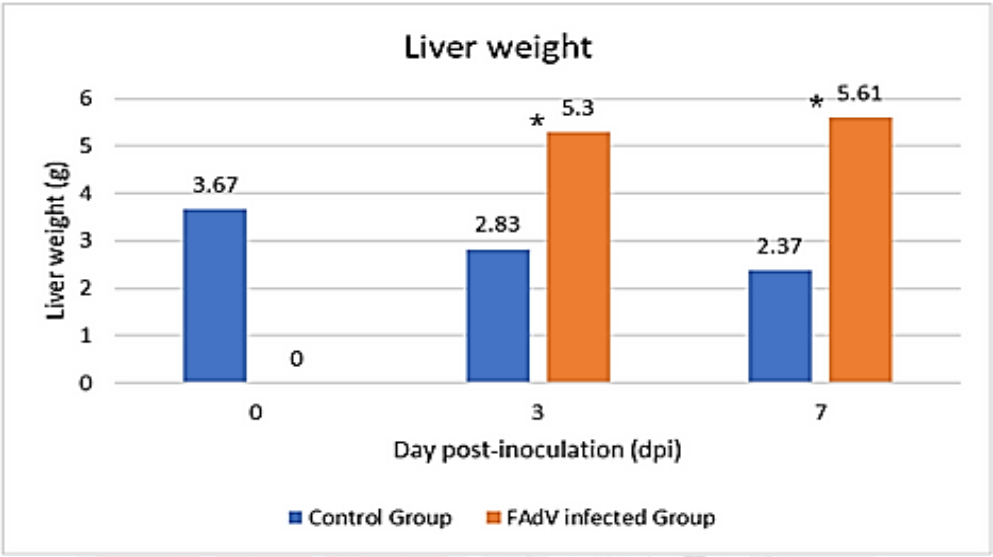

Figure 4: Mean liver weight of chickens between control and FAdV infected groups following inoculation with UPM1901 isolate at 7 day-olds. Liver weight in FAdV infected group at day 3 and 7pi were high significantly (p<0.05) compared to the control group. *asterisk indicate significant different between group.

4.1.5 Liver Weight to Body Weight Ratio

Liver to body weight ratio for FAdV infected group was significantly higher (p<0.05) at day 3pi and 7pi which were 0.07g ± 0.003 and 0.08g± 0.01 respectively as compared to the control group (Figure 5). This can be clearly described in Appendix 4, Table 4.

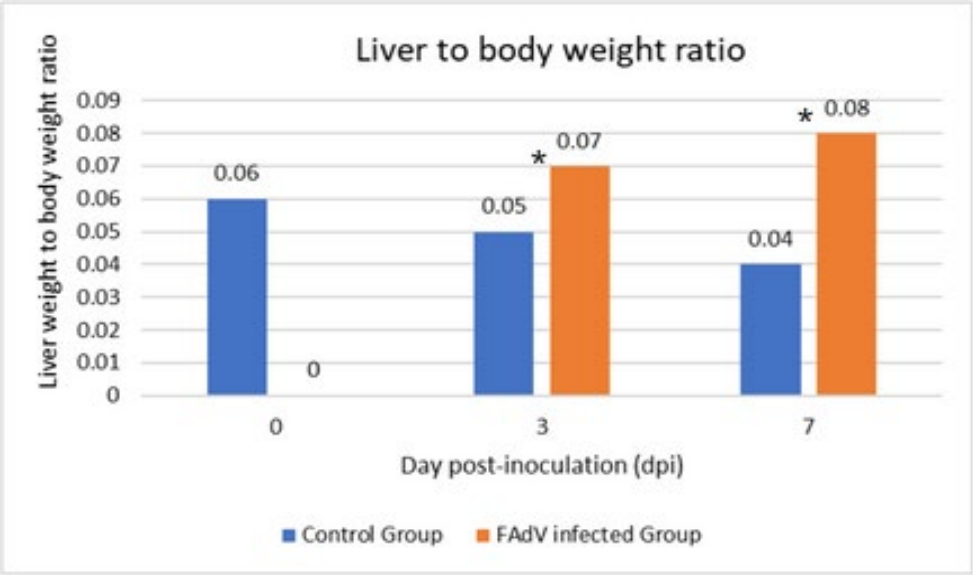

Figure 5: Mean of liver to body weight ratio between control and FAdV infected groups following inoculation with UPM1901 isolate at 7 day-olds. Liver-to-body weight ratio in FAdV infected group at day 3 and 7pi were high significantly (p<0.05) compared to the control group. *asterisk indicate significant different between group.

4.1.6 Histopathological Findings

Numerous basophilic intranuclear inclusion body (INIB) in the hepatocytes was observed at day 3 and 7pi of the infected group (Figure 6). In contrast, there is no significant finding in control group for all organs such as thymus, spleen, liver, bone marrow, ceca tonsil, bursa of Fabricius and kidney at days 3 and 7pi. For infected group, atrophy of thymus, lymphoid depletion in spleen and lymphocytic infiltration with necrosis of kidney were recorded in the infected group of chickens at day 7pi.

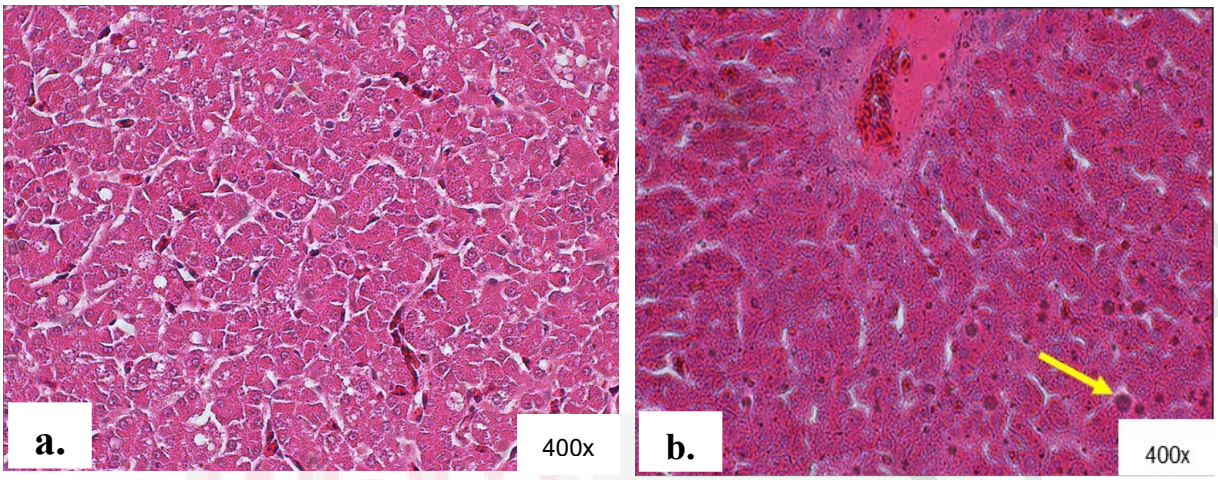

Figure 6: Histopathological findings of the liver between between control and FAdV infected groups following inoculation with UPM1901 isolate at 7 day-olds. **(a):** Normal liver in the control group, **(b):** presence of basophilic intranuclear inclusion bodies (INIB) as indicated as yellow arrow for infected group at day 3pi with H&E staining at x400 magnification.

4.1.7 Molecular Detection by Conventional Polymerase Chain Reaction (PCR)

All the tested organs including lymphoid organs, liver and kidney were positive to FAdV at days 3 and 7pi with expected PCR size product 897 base pairs (bp) (Figure 7).

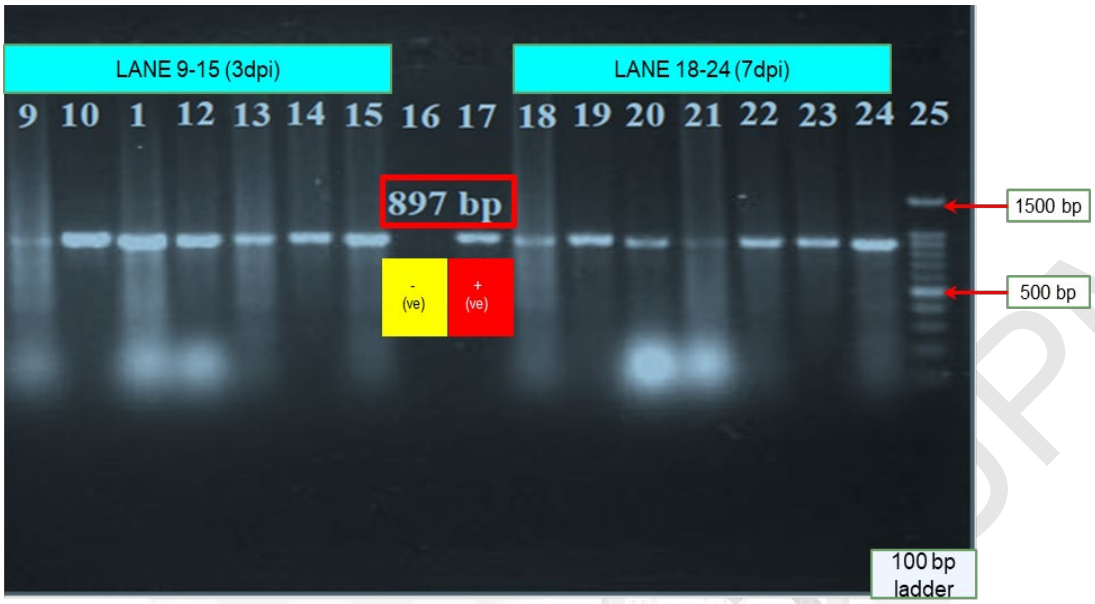

Figure 7: Electrophoresis of PCR product in 1.5% agarose gel amplifying nucleic acid of hexon gene of fowl adenovirus with expected PCR size product at 897 base pairs (bp). Lane 25: 100bp DNA marker, Lane 16: Negative control, Lane 17: Positive control, Lane 9-15: Organs from day 3 post-inoculation (pi) positive to FAdV, Lane 9: Thymus, Lane 10: Spleen, Lane 11: Liver, Lane 12: Kidney, Lane 13: Bursa of Fabricius, Lane 14: Bone Marrow, Lane 15: Cecal Tonsil, Lane 18-24: Organs from day 7pi positive to FAdV, Lane 18: Thymus, Lane 19: Spleen, Lane 20: Liver, Lane 21: Kidney, Lane 22: Bursa of Fabricius, Lane 23: Bone marrow, Lane 24: Cecal tonsil.

4.1.8 Red Blood Cells (RBC) Parameter

Red blood cells (RBC) concentration at day 7pi was significantly lower ($p<0.05$) in the infected group which was 1.6 ± 0.1 than the normal range at 2.5 – 3.5 x 10^6 µl (Figure 8). Similarly, for packed cell volume (PCV), FAdV infected group produced 18% concentration which was significantly lower ($p<0.05$) than normal range at 22 – 35% as recorded in the control group at 28% at day 7pi (Figure 9). However, there were no significant differences ($p>0.05$) at day 3pi. There were also no significant differences ($p>0.05$) for mean corpuscular volume (MCV) and

mean corpuscular hemoglobin concentration (MCHC) between control group and infected group.

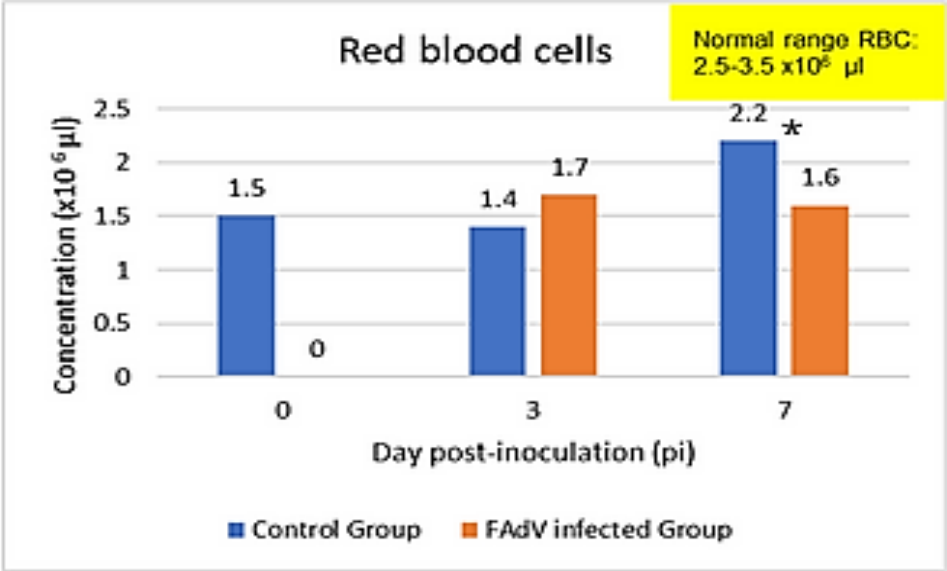

Figure 8: Concentration of red blood cells (RBC) between control and FAdV infected groups throughout the trial. RBC concentration in FAdV infected group at day 7 pi was low significantly (p<0.05) compared to control group. *asterisk indicate significant different between group.

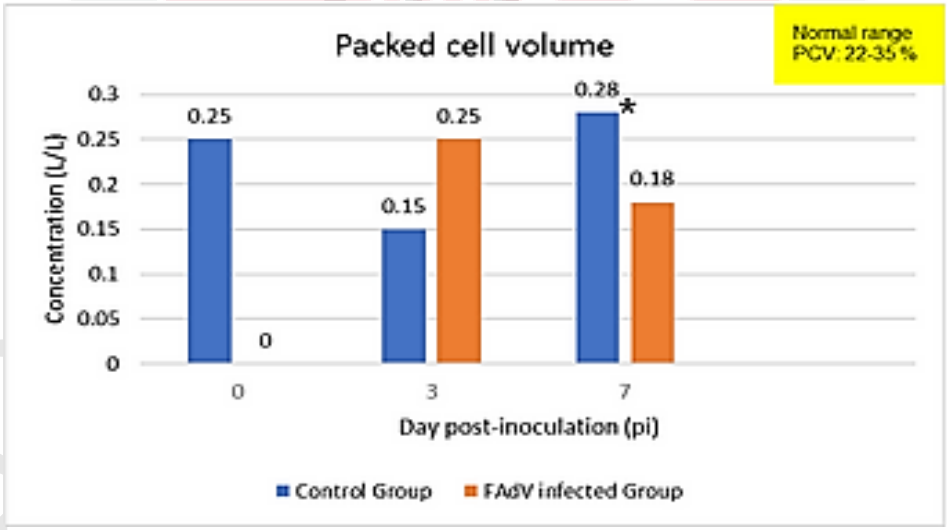

Figure 9: Mean concentration of packed cell volume (PCV) between control and FAdV infected groups throughout the trial. PCV concentration in FAdV infected group at day 7 pi was low significantly (p<0.05) compared to control group.

4.1.9 Serum Biochemistry

AST level was increased in the infected group which were 851.7 ± 436 and 795.4 ± 494 at day 3pi and 7pi, respectively than the control group at level 525.2 and 32.3 ± 88, respectively (Figure 10). However, CK level was lowered in the infected group which was 1108.2 ± 1095 at day 3pi than the control group. In addition, the CK level of the infected group was 791.5 ± 785 which was also lower as compared to the control group which was 1174.6 ± 139 at day 7pi. Lastly, the GGT level was increased in the infected FAdV group which were 15 ± 6.5 and 24 ± 4.5 at day 3pi and 7pi, respectively as compared to the control group which was 22.5 ± 11 at day 7pi.

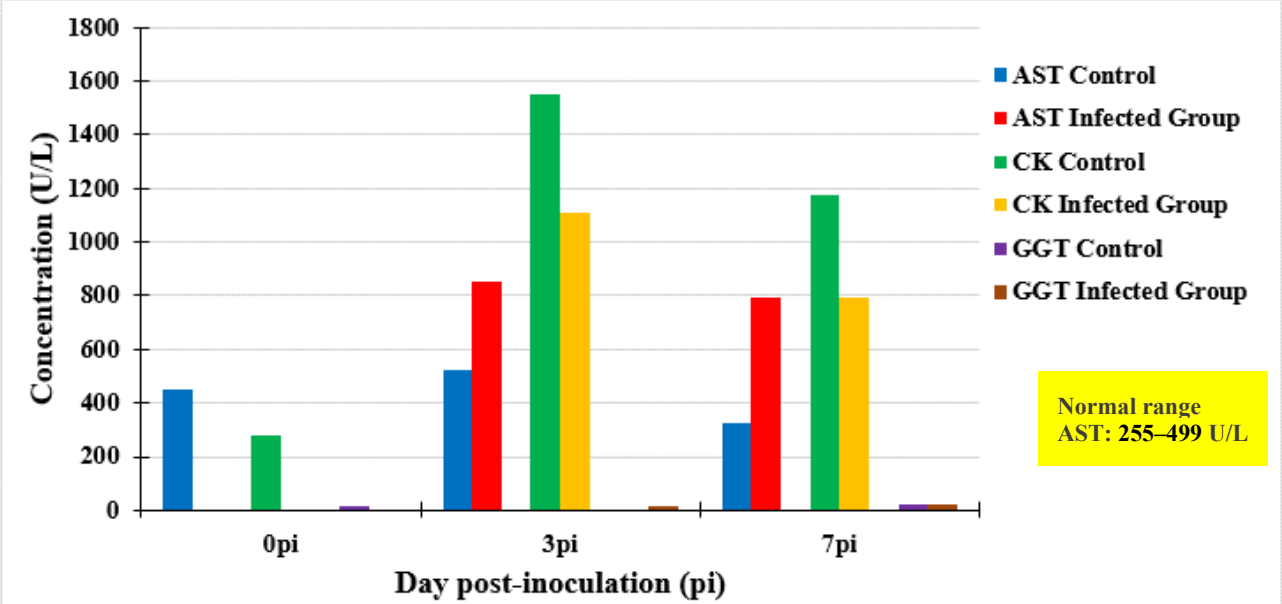

Figure 10: Mean concentration level of aspartate aminotransferase (AST), creatine kinase (CK), gamma-glutamyl transferase (GGT) enzymes between control and FAdV infected groups of chickens throughout the trial.

4.2 DISCUSSION

It was demonstrated that FAdV serotype 8b isolate UPM1901 given by oral route at day 7 old induces clinical signs shown of lethargy, depression, ruffled feathers, inappetence and watery greenish diarrhea in SPF chickens started from day 2pi onwards. The results were consistent with previous study by Cizmeicigil *et al*. (2020). In contrast, all chickens in the control group was normal throughout the trial. Mean body weight for the infected group was increased significantly (p<0.05) starting at day 3pi compared to the control group. This finding could be due to age-related resistance against FAdV as indicated by Cook *et al*. (1974) and Matos *et al*. (2016). At day 7pi, there is no significant differences (p>0.05) in body weight for both control and infected groups.

In addition, there was no mortality recorded at day 3 and 7pi in infected group, since those chickens inoculated at day 7 old. As compared to the previous study, high mortality was recorded in SPF chickens after inoculated at day old (Norfitriah *et al.*, 2019). It is believed that an evident of age-related resistance is observed with avian adenoviruses as the host's age rises, there is a notable limitation in the viral multiplication within the host, leading to a decrease in mortality (Rahimi *et al*., 2015). Another study stated that the mortality in chicken caused by IBH was peak within 3-4 days post-infection with 10% up to 30% rate of mortality (Schancher *et al.,* 2016). In addition, IBH's clinical signs and mortality peak after 3–4 days and usually return to normal on the 6th day of the outbreak (Kichou *et al*., 2019; Schachner *et al*., 2017; Hafez *et al.,* 2011).

Both gross and histopathological lesions were predominantly found in the liver as this organ was the major tropism for FAdV replication and caused severe damages. At day 3pi, the liver of the infected group was swollen, enlarged, congested and friable. The results were consistent with previous studies conducted by Sohaimi *et al*. (2019) and Norina *et al*. (2016). Moreover, there

were no abnormal findings to other tissues at day 3pi. At day 7pi, the liver of the infected group was swollen, friable, petechial hemorrhagic and necrosis which compatible with previous literature (Safwat *et al*., 2022). Besides, necropsy findings in the kidney was swollen, pale and urate deposition in the infected chickens as was reported by previous research (Tsiouris *et al*., 2022). The lesions were also similar with previous studies stating that affected chicken with IBH cause kidneys to become pale, oedematous, and mottled, enlarged with urate accumulation in the ureters (Tsiouris *et al.,* 2022).

For histopathological lesions, there was presence of numerous basophilic intranuclear inclusion body (INIB) in the hepatocytes at day 3 and 7pi of the infected group as compared to the control group. These findings were corroborated with earlier studies as described by Tsiouris *et al.* (2022) after inoculated with FAdV isolate that causes abnormality in the liver. It shown that basophilic INIB was an indicator of viral particles that existed in the liver organ. From the previous report, the presence of INIB within hepatocytes is considered as a characteristic feature of FAdV infection in IBH (Abghour *et al*., 2019; Alemnesh *et al*., 2012).

At day 7pi, the lymphoid organs showed abnormal findings which were atrophy of thymus and lymphoid depletion in the spleen. According to previous reports, there was mild loss of the lymphoid cells in the thymus of the chicken affected with IBH as in the present case which suggested that this primary lymphoid organ involved in the pathology of FAdV infection (Mirzazadeh *et al*., 2020). For the kidney, there was lymphocytic infiltration and necrosis of the kidney for the infected group at day 7pi. These findings were compatible with previous literature (Steer *et al*., 2015). Previous investigators also revealed that FAdVs can replicate in the tubular epithelium of kidneys (Wilson *et al*., 2010). However, there were no significant findings for other organs such as bone marrow, ceca tonsil and bursa of Fabricius for infected groups.

Conventional PCR was applied for this study as a molecular detection by amplifying the hexon gene. Hexon protein is the most important part of the adenovirus proteome to enable classification and recognition of individual serotypes (Ebner *et al*., 2005). This protein also plays a major role in virus infectivity and tissue tropism (Sohaimi & Bejo, 2021). The results of PCR in this study depicted that all the lymphoid organs, liver and kidney were positive to FAdV at day 3pi onwards. Based on a previous study, IBH can be classified into three stages based on the presence and severity of hepatic lesions: incubation (1–3 dpi), degeneration (4–7 dpi), and convalescence (14 dpi) (Steer *et al.,* 2015). Thus, in the present finding, the virus was present in the targeted organs from day 3pi. It showed that the virus was circulate throughout the targeted organs. This phase corresponds to the incubation period, in which fast viremia and virus multiplication was succeeded in the targeted organs (Matos *et al.,* 2016; Steer *et al*., 2015). It seems that results were consistent with gross and histological findings.

For the red blood cell (RBC) parameter, the concentration of RBC was lower at day 7pi in the infected group as compared to the control group. Similarly, packed cell volume (PCV) of infected group recorded at 18% which is indicate as anaemia compared to the control group at day 7pi. It occurs at day 7pi as a result from reduction of RBC synthesis due to affected bone marrow caused by FAdV infection. Besides, there were no significant differences (p>0.05) on RBC concentration between groups at day 3pi, perhaps, due to lysis of RBC which might be due to error during sampling or handling. For the serum biochemistry analysis, AST level was increased in the infected group at day 3 and 7pi. However, CK level was lowered in the infected group for both at day 3 and 7pi. It`s indicates that liver injury occurred in the infected group due to FAdV infection. This result was compatible with previous reported by De Luca *et al*. (2020). Although AST activity

considered very sensitive enzyme, but it is not a specific indicator for liver injury since high level of AST could be interpreted due to muscle damage as well. Thus, AST level must be compatible with CK level in order to differentiate between liver and muscle damage. For GGT level, although reference intervals have not been established, GGT values of (0 to 10 U/L) are considered normal at the Schubot Exotic Bird Health Center (College Station, Texas, USA). From the result throughout this study, the the GGT values for both control and infected group was above than the reported reference intervals (Lumeij *et al*., 1988). Increased in GGT activity is related with liver compromise and other biliary compromise such as hepatobiliary diseases (Metra *et al*., 2021; Vroon *et al*., 2012).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

FAdV-8b isolate UPM1901 causes dull, depression, diarrhoea, inappetence, lethargic and ruffled feathers in SPF chicken started from day 2pi following inoculation at day 7 old. Besides, the isolate induced gross and histological changes typically in the liver organ with swollen, friable and necrosis along with presence of basophilic intranuclear inclusion body (INIB), cellular degeneration and necrosis, respectively. Interestingly, FAdV-8b isolate UPM1901 caused anaemia in chickens and metabolic disturbances based on high level of liver enzymes. Lastly, the virus was detected in all targeted organs following infection with FAdV isolate upon performing the molecular detection via PCR.

5.2 RECOMMENDATIONS

There are several things that can be done to improve this study. Firstly, we need to extend the period of study up to 42 days to obtain a complete picture of serum biochemistry analysis. Apart from that, we should include the application of immunoperoxidase (IPS) staining for specific localization of antigen in the targeted organs. Lastly, increasing sample size is important in future to avoid any error during sampling.

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APPENDIX 1

Table 1: Experimental design for determination of metabolic disturbances and virus detection in organs of chickens infected with fowl adenovirus serotype 8b isolate UPM1901

Group / Sampling time (dpi)*	0	3	7
FAdV infected group	-	3	3
Control	3	3	3
Total SPF chickens were used in this study	**15**		

*dpi: day post-inoculation

APPENDIX 2

Table 2: Mean of body weight of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of body weight			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	83.63 ± 4.57	73.67 ± 11.38
Control	60.33 ± 1.20	58.00 ± 6.66	64.33 ± 1.76

*SEM: Standard Error Mean
[a]dpi: day post-inoculation

APPENDIX 3

Table 3: Mean of liver weight of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of Liver Weight			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	5.30 ± 0.09	5.61 ± 0.58
Control	3.67 ± 0.67	2.83 ± 0.23	2.37 ± 0.12

*SEM: Standard Error Mean
[a]dpi: day post-inoculation

APPENDIX 4

Table 4: Mean of liver weight to body weight ratio of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of liver weight to body weight ratio			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	0.07 ± 0.003	0.08 ± 0.010
Control	0.06 ± 0.01	0.05 ± 0.003	0.04 ± 0.003

*SEM: Standard Error Mean
[a]dpi: day post-inoculation

APPENDIX 5

Table 5: Mean bursa of Fabricius weight of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of Bursa of Fabricius eight			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	0.30 ± 0.02	0.09 ± 0.03
Control	0.05 ± 0.00	0.14 ± 0.01	0.14 ± 0.03

*SEM: Standard Error Mean
[a]dpi: day post-inoculation

APPENDIX 6

Table 6: Mean aspartate aminotransferase (AST) level of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of aspartate aminotransferase (AST) level			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	851.7 ± 436	794.4 ± 494
Control	435.6 ± 149	525.2	323.3 ± 88

*SEM: Standard Error Mean
[a]dpi: day post-inoculation

APPENDIX 7

Table 7: Mean creatine kinase (CK) level of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of creatine kinase (CK) level			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	1108.2 ± 1095	791.5 ± 785
Control	282.5 ± 156	1553.9	1174.6 ± 139

*SEM: Standard Error Mean
[a]dpi: day post-inoculation

APPENDIX 8

Table 8: Mean gamma-glutamyltransferase (GGT) level of chickens in control groups and FAdV infected groups throughout the study

Mean ± SEM* of gamma-glutamyltransferase (GGT) level			
Group / Sampling time (dpi)[a]	0	3	7
FAdV infected group	0	15 ± 6.5	24 ± 4.5
Control	14 ± 1.5	NA*	22.5 ± 11

*SEM: Standard Error Mean
*NA: not available
[a]dpi: day post-inoculation