



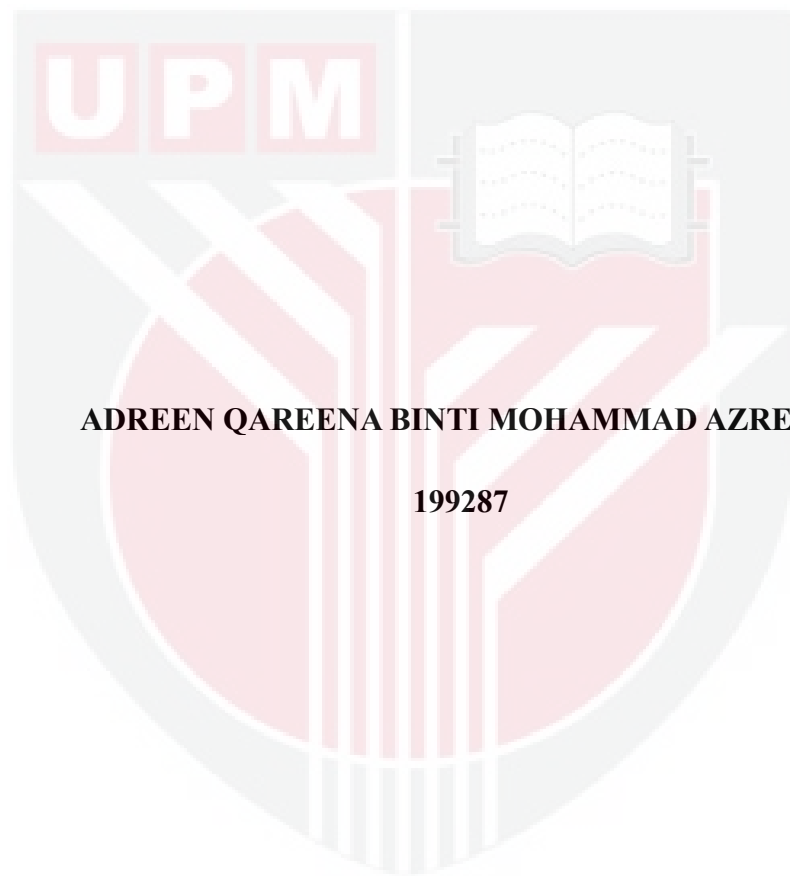
UNIVERSITI PUTRA MALAYSIA

**SAFETY AND IMMUNOGENICITY OF INACTIVATED FOWL
ADENOVIRUS FOLLOWING DIFFERENT INACTIVATION TIME
INTERVALS IN BROILER CHICKENS**

ADREEN QAREENA BINTI MOHAMMAD AZREEN

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FPV 2022 68**

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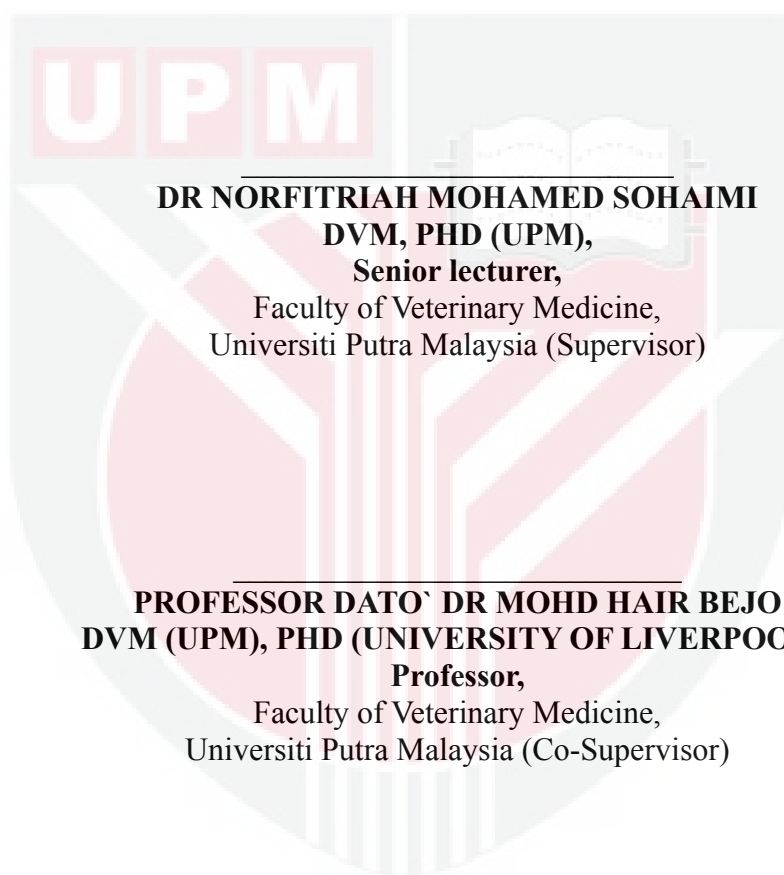
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**Dissertation submitted in partial fulfilment of the requirement for the course
VPD4999- Final Year Project in the Department of Veterinary Clinical Studies
Universiti Putra Malaysia**

OCTOBER 2022

CERTIFICATION

It is hereby certified that we have read this project paper entitle “ Safety and Immunogenicity of Inactivated Fowl Adenovirus Following Different Inactivation Time Interval in Broiler Chickens by Adreen Qareena Binti Mohammad Azreen 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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TABLE OF CONTENT

CERTIFICATION	i
ACKNOWLEDGEMENTS	ii
LIST OF FIGURES	vi
LIST OF APPENDICES	vii
ABBREVIATIONS	viii
ABSTRAK	ix
ABSTRACT	xi
CHAPTER 1	1
1 INTRODUCTION	1
1.1 Background	1
1.2 Justification	2
1.3 Hypothesis	3
1.3 Objectives	3
2 LITERATURE REVIEW	4
2.1 Aetiology	4
2.1.1 Classification	
2.1.2 Morphology	
2.2 Epidemiology	5
2.2.1 Mode of Transmission	
2.3 Pathogenesis	6
2.4 Clinical Signs	6
2.5 Pathology	6
2.5.1 Gross Lesions	
2.5.2 Histopathological Lesions	
2.6 Diagnosis	7
2.6.1 Virus Isolation and Identification	
2.6.2 Serology	
2.7 Control and Prevention	8
2.8 Inactivated Fowl Adenovirus Vaccine Potential	9
2.9 Methods of Inactivation of Virus	10
2.10 Current Situation of Fowl Adenovirus in Malaysia	11
3 MATERIALS AND METHODS	12
3.1 Experimental Design	12
3.2 Inactivated FAdV	13
3.3 Gross Pathology	13
3.4 Histopathology	13
3.5 Enzyme Linked Immunosorbent Assay	14
3.6 Statistical Analysis	15
4 RESULTS	16

4.1	Clinical Signs	16
4.2	Body Weight	16
4.3	Gross Lesions	17
4.4	Histopathological Lesions	18
4.5	Antibody Titer	19
5	DISCUSSION, CONCLUSION AND RECOMMENDATION	21
5.1	Discussion	21
5.2	Conclusion	30
5.3	Recommendation	30
	REFERENCES	31
	APPENDICES	43



LIST OF FIGURES

Figures	Caption	Page
Figure 1	Body weight of chickens between inoculated (Group A and B) and control groups throughout the trial.	17
Figure 2	Necropsy finding of liver in chickens between booster and non-booster groups following vaccination with inactivated FAdV at 36h (group A) and 32h (group B) at day 28pi.	17
Figure 3	Histological finding of liver between inoculated (Group A and B) and non-inoculated (Control) groups at day 28pi.	18
Figure 4	FAdV antibody titer of chickens between inoculated and control groups throughout the trial.	20

LIST OF APPENDICES

Appendix	Caption	Page
1	Experimental design for safety and immunogenicity study of inactivated FAdV UPM1137	43
2	Body weight of chickens throughout the trial	44
3	FAdV antibody titer of chickens throughout the trial	44



ABBREVIATIONS

BEI	Binary ethylenimine
CEL	Chicken embryo liver
FAdV	Fowl adenovirus
FAdV-4	Fowl adenovirus serotype 4
HPS	Hydropericardium syndrome
HHS	Hepatitis-hydropericardium syndrome
IBH	Inclusion Body Hepatitis
EDS	Egg Drop Syndrome
ELISA	Enzyme-linked Immunosorbent assay
(h)	hours

ABSTRAK

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

KESELAMATAN DAN KEIMUNOGENAN ADENOVIRUS UNGGAS YANG TIDAK AKTIF MENGIKUT SELANG MASA PENYAHAKTIFAN YANG BERBEZA DALAM AYAM PEDAGING

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Dalam beberapa tahun kebelakangan ini, banyak perhatian telah ditumpukan kepada pembangunan vaksin Adenovirus unggas yang tidak aktif (FAdV) untuk industri ayam Malaysia menentang wabak “inclusion body hepatitis” (IBH). Objektif kajian ini adalah untuk menentukan keselamatan dan keimmunogenan vaksin FAdV yang tidak aktif mengikut selang masa penyahaktifan yang berbeza dalam ayam pedaging komersial. Berdasarkan penemuan semasa, ia telah menunjukkan bahawa tanda-tanda klinikal, mahupun lesi kasar dan histologi tidak direkodkan dalam semua kumpulan sepanjang percubaan. Vaksin ini selamat tanpa menjejaskan prestasi ayam

sepanjang percubaan kerana berat badan untuk semua kumpulan tidak perbezaan ketara ($p > 0.05$) pada hari ke-14 dan 28 pasaca-infeksi (pi). Berdasarkan penemuan ELISA, ayam yang divaksin dengan FAdV yang tidak aktif pada 32 jam (j) (kumpulan B) mencetuskan titer antibodi yang tinggi berbanding dengan FAdV yang tidak diaktifkan pada 36j (kumpulan A) pada hari ke-14 dan 28pi. Pada hari 28pi, purata titer antibodi bagi yang tiada penggalak, kumpulan A1 (321 ± 189) dan B1 (602 ± 367) adalah tinggi dengan ketara ($p < 0.05$) berbanding kumpulan kawalan (1 ± 0). Begitu juga, titer antibodi untuk kedua-dua kumpulan penggalak, A2 (690 ± 484) dan B2 (874 ± 317), adalah tinggi dengan ketara ($p < 0.05$) berbanding kumpulan kawalan (1 ± 0). Ia menunjukkan bahawa kedua-dua vaksin FAdV menggunakan inokulum tidak aktif pada 32j dan 36j adalah selamat dan imunogenik dalam ayam pedaging terutamanya pada hari 28pi selepas vaksinasi penggalak. Disimpulkan bahawa tempoh optimum penyahaktifan FAdV adalah 32 jam dan sesuai digunakan untuk formulasi vaksin pada masa hadapan.

Kata kunci: Adenovirus unggas (FAdV); 'inclusion body hepatitis' (IBH); vaksin; keselamatan; keimunogenan

ABSTRACT

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

SAFETY AND IMMUNOGENICITY OF INACTIVATED FOWL ADENOVIRUS FOLLOWING DIFFERENT INACTIVATION TIME INTERVAL IN BROILER CHICKENS

By

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Dr. Nor Yasmin Abd. Rahaman

In recent years, much attention has been focused on the development of inactivated fowl adenovirus (FAdV) vaccines for the Malaysian poultry industry against inclusion body hepatitis (IBH) outbreak. It was the objective of this study to determine the safety and immunogenicity of inactivated FAdV following different inactivation time intervals in commercial broiler chickens. It was demonstrated that neither clinical signs, nor gross and histological lesions were recorded in all groups throughout the trial. The vaccine is safe without affecting the chicken performance throughout the trial since the body weight for all groups was no significant difference ($p>0.05$) at day 14 and day 28 post inoculation (pi). Based on ELISA findings, chickens vaccinated with inactivated FAdV at 32 hours (h) (group B) induce high

antibody titer compared to that inactivated FAdV at 36h (group A) at day 14 and 28pi. At day 28pi, mean antibody titer for non-booster, groups A1 (321 ± 189) and B1 (602 ± 367) were significantly high ($p < 0.05$) as compared to the control group (1 ± 0). Similarly, antibody titer for both booster groups, A2 (690 ± 484) and B2 (874 ± 317), are significantly high ($p < 0.05$) compared to the control group (1 ± 0). Both FAdV vaccines using inactivated inoculum at 32h and 36h are safe and immunogenic in broiler chickens mainly at day 28pi following booster vaccination. It was concluded that the optimal period of FAdV inactivation was 32 hours and suitable to be used for future vaccine formulation.

Keywords: Fowl adenovirus (FAdV); inclusion body hepatitis (IBH); vaccine; safety; immunogenicity.

CHAPTER 1

INTRODUCTION

Background

Fowl adenovirus (FAdV) infection is a major threat in commercial poultry farms and has a serious economic impact on the poultry industry. Previously, it was reported that a decrease of 9.0% in revenue to RM 692.9 million in the end year of 2018 due to high mortality and low broiler production volume resulting from inclusion body hepatitis (IBH) outbreaks. An outbreak of IBH was first reported in Perak in 2005. Since then, the number of IBH cases has continued to increase in recent years due to unavailable suitable vaccines against the disease (Sohaimi *et al.*, 2019). Thus, it urgently needs an appropriate vaccine to control disease outbreaks in the poultry farm.

FAdV, which infects poultry species, belongs to the Aviadenovirus genus under the family of Adenoviridae and consists of five molecular species known as species A to E and divided further into 12 serotypes. FAdV caused several clinical diseases in chickens with significant economic impact due to mortality and poor productivity (Cizmecigil *et al.*, 2020; Cui *et al.*, 2020). IBH was reported worldwide mainly in meat producing chickens at 2 to 3-week old age and some in-layer chickens ranged from 25 to 27-week old (Abghour *et al.*, 2019; Norfitriah *et al.*, 2018). Epidemiological studies confirmed that IBH is commonly caused by serotypes 2, 8a, 8b and 11 (Schachner *et al.*, 2018). While, hepatitis-hydropericardium syndrome (HHS) and gizzard erosion are mainly due to serotypes 1 and 4. In Malaysia, IBH and gizzard erosion were reported recently in broiler and layer chickens due to FAdV serotype 8b infection (Sohaimi *et al.*, 2019). The isolate is highly pathogenic in SPF

chickens under the experimental condition, with 100% mortality and severe lesions in the liver (Norfitriah *et al.*, 2019).

The application of the vaccine in other countries is capable of controlling virus spreading at vertical and horizontal levels (Alvarado *et al.*, 2007). Several studies on vaccine development were conducted extensively against serotype 4 due to the lack of commercialized vaccines. As reported in China, the inactivated vaccine conferred full protection against novel FAdV serotype 4 (Pan *et al.*, 2017). In recent work, bivalent live and inactivated vaccines can protect chicks against IBH through breeder vaccination (Gupta *et al.*, 2018). The other serotype 2 inactivated vaccine was also developed in Thailand and provides 90 to 100% protection in progeny stocks by vaccination in broiler breeders (Junnu *et al.*, 2015). As reported in early studies, chemical treatment by formalin and binary ethyleneimine (BEI) are commonly used chemicals for virus inactivation in poultry vaccine production (Lone *et al.*, 2017). In vaccinated chickens, membrane alteration in formalin's FAdV particles caused poor immunogenicity compared to inactivated virus suspension by BEI (Mansoor *et al.*, 2011).

Justification

Malaysia amongst the top global consumers of poultry meat worldwide with 63kg meat consumption per capita in 2019 (Poultry World, 2020). However, inclusion body hepatitis (IBH) caused by fowl adenovirus (FAdV) serotype 8b has been a major threat to the poultry industry in recent years with significant economic losses due to high mortality and poor production in commercial farms. Thus, proper biosecurity and vaccination are crucial to sustain food security in the country. The number of clinical cases of IBH continued to increase in recent years due to

unavailable local vaccines against high pathogenic FAdV serotype 8b in commercial chickens. Development of suitable inactivated vaccines necessitates knowledge on optimum virus inactivation which remain scanty for FAdV regardless of serotype and strains.

Hypothesis

The null hypothesis (H_0) was there is no difference on safety and antibody response level regardless of the different time of FAdV inactivation and the dose in broiler chickens. The alternate hypothesis (H_A) was that the different time interval of FAdV inactivation at 32h is the optimum period of inactivation and safe to induce high FAdV antibody titer with the booster dose in broiler chickens.

Objective

The objective of this study is:

- 1) to determine safety and immunogenicity of inactivated fowl adenovirus (FAdV) following different inactivation time intervals in broiler chickens
- 2) to evaluate the FAdV antibody titer between booster and non-booster groups in the vaccinated broiler chickens.

CHAPTER 2

LITERATURE REVIEW

2.1 Aetiology

2.1.1 Classification

Fowl adenovirus (FAdV) is classified to the family *Adenoviridae*, genus *Aviadenovirus* and are divided into 5 groups (A-E) and into 12 serotypes which are FAdV-1 to 8a, 8b, 9, 10 and 11 (Hess, 2000).

2.1.2 Morphology

Adenoviruses are icosahedral non-enveloped double-stranded DNA viruses. They are 74–90 nm in size and have a characteristic morphology (Smyth & McNulty, 2008). The nucleic acid is linear, double-stranded deoxyribonucleic acid (McFerran and Smyth, 2000). The capsomeres seemed elongated and were arranged in facets of equilateral triangles as described for human adenovirus, according to Horne *et al.* (1959). The icosahedral symmetry of individual particles was indicated by their hexagonal profiles. Six capsomeres were calculated to occupy each facet edge, giving the viral capsid a total of 252 capsomeres. According to Harrach (2014), aviadenovirus virions have two fibres per vertex. Two fibre genes and two projections with noticeably differing lengths from each penton base are present in fowl adenovirus 1 and 4 (FAdV-1 and -4). There are two fibres per vertex in other fowl adenoviruses as well, but there appears to be just one fibre gene, therefore the projections are similar in length.

2.2 Epidemiology

2.2.1 Mode of Transmission

According to McFerran & Adair (1977), fowl adenoviruses are readily transmitted horizontally since they are present in all excretions, especially in the feces. Fomites, personnel, and transport significantly contribute to the spread of the virus. According to McCracken and Adair (1993), vertical transmission is reported as a very efficient way to spread from parent bird to progenies. Adenovirus is transmitted through embryonated eggs. Reports established of latent infection with FAdV in chickens (Grgić *et al.*, 2006).

2.3 Pathogenesis

It is well known that avian adenoviruses are widely distributed throughout the host's internal organs and can cause from mild to major diseases. The pathogenicity of different strains varies (Sharma & Adlakha, 2009). However, most of the strains perform the same pattern of infection according to McFerran & Adair (1977). The exposure to other microbial agents can increase the severity of the disease. Based on McFerran & Adair (1977) they claimed that following initial multiplication at the site of virus entry there is viraemia resulting in virus spread to virtually all organs. The blood brain barrier normally prevents entry of viruses into the central nervous system. The main sites of virus replication appear to be in the trachea and caeca. According to Smyth & McNulty (2008), Aviadenoviruses can establish latent infections and infected chickens can become potential lifelong carriers and more likely to shed the virus throughout their period of life.

2.4 Clinical Signs

Aviadenovirus are commonly associated with inclusion body hepatitis (IBH), hydropericardium syndrome, respiratory disease, egg drop syndrome (EDS), diarrhea, tenosynovitis, poor growth and reduced feed conversion (Smyth &

McNulty, 2008). According to research done by Asrani *et al.* (1997), clinical signs of chicken infected with the inclusion body hepatitis (IBH) and hydropericardium syndrome typically showed signs of dullness and depression, ruffled feathers with their chest close to the ground. Incubation period can be up to 24 - 48 hours (McFerran and Adair 2003). According to Smyth & McNulty (2008), inclusion body hepatitis (IBH) can be seen in broiler chickens aged 3 to 7 weeks but can also be seen in younger and older birds. Inclusion body hepatitis (IBH) is characterized by a rapid disease course of 1 to 3 weeks, diarrhea, decreased appetite, lethargy, and low to moderate mortality rates of less than 10% to 30% (McFerran and Adair 2003).

2.5 Pathology

2.5.1 Gross Lesions

The gross lesions upon necropsy include mild to moderate enlargement of liver with pale, friable and fatty change appearance and areas of hemorrhages and congestion as well as pale and slightly enlarged kidney (Hair-Bejo, 2005). In cases of hydropericardium syndrome, the hearts were flabby with amber-colored and jelly-like fluid in the pericardial sacs (Ren *et al.*, 2019). Gross pathology also resulted in lungs and kidneys being enlarged with edema and congestion (Sun *et al.*, 2019). According to Lim *et al.* (2012) focal gizzard lesions such as rough or cloudy koilin layer can be observed .

2.5.2 Histological Lesions

Histologically, the most prominent lesion is inclusion body hepatitis which can be found in the liver with presence of large, round eosinophilic or basophilic viral inclusion bodies in the hepatocytes. Besides, another lesion can be found is tracheitis with presence of lymphocytes infiltration in the trachea (Timmerman *et al.*, 2013). In

cases of hydropericardium syndrome, the heart muscle fibers were fractured with extensive congestion and hemorrhages (Ren *et al.*, 2019). According to recent research, histological findings concluded that interstitial vascular dilation and widening of the gap between myocardial fibers became more severe over time (Sun *et al.*, 2019). In cases of gizzard erosion, severe degeneration and necrosis of glandular epithelium and eosinophilic inclusion bodies were observed under microscopic examination (Lim *et al.*, 2012).

2.6 Diagnosis

2.6.1 Virus Isolation and Identification

Diagnosing aviadenovirus typically on clinical signs is insufficient. Histological investigation can be done by staining of suspected cells with hematoxylin and eosin to demonstrate typical intranuclear lesion (Gallina *et al.*, 1973). According to Smyth & McNulty (2008), virus isolation can be done by inoculating both a 10% suspension of the affected organ and a faeces suspension into cell cultures especially in chick embryo liver or lung cells, or chick kidney cells since they are considered to be more sensitive. Virus identification can be achieved by virus neutralization test (VNT) or fluorescent antibody test. The serum antibodies can be detected by gel diffusion or by virus neutralization test (Sharma & Adlakha, 2009). In recent years, more rapid and sensitive tests such as polymerase chain reaction (PCR) and sequencing based on hexon and fiber genes were performed for diagnosis of FAdV serotypes in affected farms (Sohaimi *et al.*, 2018).

2.6.2 Serology

The enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence has been used to distinguish group-specific antibodies as it is inexpensive and more

sensitive (McNulty & Smyth, 2002). Since the efficacy of inactivated vaccines is related to the induction of humoral immune responses, antibody levels in vaccinated chickens may be a useful measure for evaluating inactivated vaccines (Kim *et al.*, 2014). Based on several studies, they observed increased antibody levels against FAdV after inactivated FAdV-4 vaccination. According to previous study, mono-serotype of FAdV antigen alone could broadly react with antiserum of other serotypes of FAdV in ELISA (Calnek *et al.*, 1982; Saifuddin & Wilks, 1990).

2.7 Control and Prevention

In cases of outbreaks, there is no specific treatment available against this viral disease; however antibiotics are used for prevention of secondary bacterial infections according to Merck's Manual (2011). According to Chisti *et al* (1989), disinfection, maintaining biosecurity and proper ventilation can significantly reduce the chance of infection. Akhtar *et al* (2000) also mentioned that proper disinfection of premises and equipment, restricted entry of visitors and vaccination crews and ventilation and proper lighting in the poultry houses play a significant role in prevention of the disease. Based on few reports, adding iodophor solution to drinking water has been effective in reducing mortality in broiler farms (Abdul-Aziz and Al-Attar 1991; Abdul Aziz and Hasan, 1996). IBH was initially controlled by using an autogenous vaccine which is using an inactivated liver homogenate vaccine that was formulated after the first outbreak. It showed that the vaccine had successfully prevented the disease and reduced the mortality rates (Chisti *et al.*, 1989; Akhtar *et al.*, 2000; Ahmad and Hasan; 2004). Vaccination is most effective in managing disease as a prevention. Based on Afzal and Ahmad (1990), the mortality in vaccinated infected chickens is lower compared to unvaccinated infected chickens. Safe vaccines can

induce strong active immunity to protect broiler chickens throughout their growth period (Balamurugan & Kataria, 2004).

2.8 Inactivated Fowl Adenovirus Vaccine Potential

Inactivated vaccines are convenient to prepare and store. Inactivated vaccines of a virus can alter viral antigens, reducing the safety or efficacy of the vaccine. Ideally, the conditions and agents for viral inactivation would selectively and irreversibly affect the viral genome (Budowsky *et al.*, 1999). According to practical experience, a single or double dose of an inactivated vaccine has been effective in managing the disease (Junnu *et al.*, 2015). However, research has established that there is still potential biosafety risk on inactivated vaccines (Su *et al.*, 2020). Since the wild-type virulent strain of FAdV has been used to make all FAdV-inactivated vaccine candidates to date, a safer vaccine strain is critically required in order to produce novel vaccines and lower biosafety risks (Zhang *et al.*, 2022). Also according to the study done by Zhang *et al.* (2022), the first inactivated FAdV-4 vaccine using a non-pathogenic strain has been developed. This will remarkably reduce the potential biosafety risks in vaccine production and clinical immunization. Study conducted by Kim *et al.* (2014) demonstrated that an inactivated vaccine composed of FAdV-4 alone could provide broad cross-protection against various serotypes of FAdVs. Based on the study, more than one serotype of FAdV may be present on a farm and would be difficult to monitor less pathogenic serotypes of FAdV. Therefore, FAdV vaccines that provide broad cross-protection are considered effective against the disease (Balamurugan & Kataria, 2004).

2.9 Methods of Inactivation of Fowl Adenovirus

There are many types of methods of inactivation of viruses but in this literature review, it is more focused on the methods used to inactivate FAdV specifically. According to studies conducted by Kumar *et al.*, (1997) and Kanwar *et al.*, (2010), the FAdV-4 isolate was inactivated by heating under different types of temperatures (at 60°C for 1 hour, 80°C for 10 minutes, and 100°C for 5 minutes). By heating the virus, the virus can become inactivated by the denaturation process of the secondary or tertiary viral structure. It is possible the confirmation of viral proteins for attachment and replication of host cells is changed in this method (Delrue *et al.*, 2012). Afzal *et al.* (1990) demonstrated the inactivation of FAdV-4 isolate under different ranges of pH. Inactivation of a virus at different ranges of pH is also a type of denaturing process which affects the protein. A low (acidic) or a high (alkaline) pH can inactivate viruses via denaturation of viral proteins that are involved in attachment and replication of host cells (Delrue *et al.*, 2012).

Several previous vaccine development studies for the control and prevention of IBH and HHS disease have either been attenuated adaptation vaccine (14-16 passages) or inactivated vaccine treated with chemicals such as formalin and binary ethyleneimine (BEI). According to a study conducted by Akhtar *et al.*, (2000), BEI inactivated vaccines performed better than formalin inactivated vaccines in terms of antibody titer. BEI is an alkylating agent and used for virus inactivation by blocking the genome reading and reacts very little to proteins (Delrue *et al.*, 2012). Therefore, the antigenic components of the virus are not altered (Akhtar *et al.*, 2000). However, formalin affects the protein membrane and reduces the antigenicity of an antigen. These effects can also cause disease in vaccinated chickens (Bahnemann, 1990). Formaldehyde can destroy the viral structure which leads to poor immune response (Delrue *et al.*, 2012).

2.10 Current Situation of Fowl Adenovirus in Malaysia

Fowl adenovirus has become a major problem in commercial poultry farms mainly due to it significantly affecting the economical aspect of the poultry industry. Malaysia is amongst the top global consumer poultry meat worldwide with 44 kg meat consumption per capita in 2021 (Statista Research Department, 2022). It is reported that FAdV serotype 8b has predominantly affected the commercial chickens in Malaysia with inclusion body hepatitis (IBH) and gizzard erosion which has caused high mortality and poor production in commercial farms (Norina *et al.*, 2016; Sohaimi *et al.*, 2019). The number of IBH cases has continued to increase over the years due to absence of developed local vaccines against high pathogenic FAdV serotype 8b in commercial chickens (Sohaimi *et al.*, 2019; Sabarudin *et al.*, 2021). Therefore, proper biosecurity and development of vaccines has primarily been the focus to sustain the food security in the country.

CHAPTER 3

MATERIALS AND METHODS

3.1 Inactivated FAdV inoculum

FAdV isolate namely, UPM1137CEL5, at fifth passage in primary CEL cells at virus titer $10^{12.3}$ TCID₅₀/ml was inactivated with 0.002M concentration of binary ethyleneimine (BEI) at 6 different intervals time, 20 hours (h), 24h, 28h, 32h and 36h. Safety tests in the SPF chicken embryonated eggs revealed 100% embryonic mortalities were recorded following inoculation by inactivated FAdV inoculums at 20h, 24h and 28h. For isolate inactivated at 32h and 36h, no mortality was recorded throughout the trial. For vaccine preparation, the inactivated FAdV inoculum at 32h and 36h will be used in this study. Montanide ISA 71 VG (SEPPIC, Inc.) were used as an adjuvant in this trial with ratio 70:30 (adjuvant : antigen) (v/v). Each virus suspension at 32h and 36h will be mixed with adjuvant accordingly and stored in a separate tube (Norfitriah, personal communication, September 21, 2022).

3.2 Source of chicks

Forty-five (45) one-day old Cobb 500 commercial broiler chickens were purchased from a hatchery in Rembau, Negeri Sembilan. All chicks were kept in the Animal Research Facility (ARF) in the Faculty of Veterinary Medicine, Universiti Putra Malaysia.

3.3 Experimental Design

The study was conducted under IACUC approval number: UPM/IACUC/AUP-U012/2022. All chicks were reared in wire-floored isolated house with given food and water ad-libitum. The chicks were divided into 3 major

groups namely A, B and C. Fifteen (15) chickens were assigned in individual groups A and B, while fifteen (15) chickens in group C. Each group A and B will be divided further into booster (A1 and B1) and non-booster groups (A2 and B2). All chicks in groups A1 and A2 were inoculated with 0.2ml vaccine prepared from inactivated FAdV inoculum at 36h, while group B1 and B2 received 0.2ml vaccine prepared from inactivated FAdV inoculum at 32h. Inoculation was conducted via subcutaneous (SQ) route (0.2ml) using vaccine formulated with Montanide ISA 71 VG at vaccine dose 10^8 TCID₅₀/ml on day old via subcutaneous (SQ) route. All chicks in group C remained uninoculated and were used as the control group.

At day 14 post-inoculation (pi), all chicks in groups A2 and B2 received a booster at 0.2ml vaccine accordingly via SQ route. All chicks were monitored daily for clinical signs at least twice daily. At day 0pi, five (5) chicks were sacrificed in group C (Control) through cervical dislocation. At day 24pi, five chicks were sacrificed in group A1, B1 and C through cervical dislocation. At day 28pi, all chicks were sacrificed from all groups through cervical dislocation. The body weight and serum sample were collected from each chicken prior to sacrifice (**Appendix 1, Table A1**).

3.4 Gross Lesions

Gross lesions of the chicken were observed and recorded during necropsy. Both liver and trachea were collected for histological examination (Sohaimi *et al.*, 2018).

3.5 Histopathology

Liver and trachea from each chicken were collected during the necropsy and were fixed immediately in 10% neutral buffered formalin in the specimen container for at least 24 hours. After fixation, tissue trimming was done to create an even and flat surface in the area of interest before placing it into the cassette. Liver in one cassette

and trachea in a separate cassette with longitudinal and circular sections cut off. Second, tissue processing which involves dehydration with a series of alcohol 70% to 90% to 100% and clearing with xylene. The cassettes were placed into an automated tissue processor for 16 hours. Next, the tissue embedding was done where the tissue was oriented properly in the mould filled with paraffin wax. Once the tissue had been embedded. They were trimmed and sectioned to 4 micrometers using a microtome. After the sections were cut, they were floated on a warm water bath that helps to remove wrinkles. The sections were then mounted on glass slides, followed by heating which helped to adhere the section to the slide. Lastly, the slides undergo dewaxing and are followed by Hematoxylin and Eosin (HE). The tissues were then carefully examined under microscope using x4, x10, x20, x40 and x100 objectives for histological changes (Toro *et al.*, 2002).

3.6 Enzyme Linked Immunosorbent Assay

All serum samples were collected and analysed for FAdV antibody titer using enzyme linked immunosorbent assay (ELISA) technique. The enzyme linked immunosorbent assay (ELISA) test was carried out according to the methods described by BioChek, London, UK. Each test sample was diluted to 1:100 in sample diluent reagent. FAdV Gp1 coated plate was removed from the sealed bag and the location of samples were recorded on template. Negative control (100 μ L) was added into wells A1 and B1. Positive control (100 μ L) was added into wells C1 and D1. Reference control was added into E1 and F1. Diluted samples (100 μ L) were added into appropriate samples. The plate was covered with a lid and incubated at room temperature for 30 minutes. The contents of wells were aspirated and washed 4 times with a wash buffer (350 μ L per well) . The plate was inverted and tapped firmly on absorbent paper until no moisture was visible. Conjugate reagent (100 μ L) was

added into the appropriate wells. The plate was covered with a lid and incubated at room temperature for 30 minutes. The wash procedure was repeated. Substrate reagent (100 μ L) was added into appropriate wells. Stop solution (100 μ L) was added into appropriate wells to stop the reaction. The microtiter plate reader was blanked on air and the absorbance of control and the samples were recorded by reading at 405 nm.

3.7 Statistical Analysis

Mean body weight and FAdV antibody titers data were analysed with one-way analysis of variances (ANOVA) using IBM SPSS Statistics version 22. The significance level was determined at alpha value, $p < 0.05$. For the following significant outcome, multiple group comparison tests were performed using Tukey HSD (Honest Significant Difference) test. Independent t-test was conducted to compare between two group means (Field, 2013).

CHAPTER 4

RESULTS

4.1 Clinical Signs

All the chickens were normal, feeding and drinking well. No abnormal clinical signs of FAdV and no mortality was observed in all groups of chicken throughout the trial.

4.2 Body Weight

The body weight of the day old chicks was 0.074 ± 0.003 kg. The body weight of chicken on day 14 pi from Control Group, Group A and Group B were 0.753 ± 0.017 kg, 0.7 ± 0.027 kg and 0.678 ± 0.022 kg, respectively (**Appendix 2, Table A2**). After 28 days of trial, the body weight of the chickens in groups control, A1, A2, B1 and B2 were 1.888 ± 0.101 kg, 1.646 ± 0.165 kg, 1.893 ± 0.061 kg, 1.813 ± 0.06 kg and 2.103 ± 0.027 kg, respectively (Figure 1). There is no significant difference ($p > 0.05$) in body weight between all groups at day 14 pi and day 28 pi. There is also no significant difference ($p > 0.05$) in body weight between booster (Group A1 and B1) and non-booster (Group A2 and B2) groups.

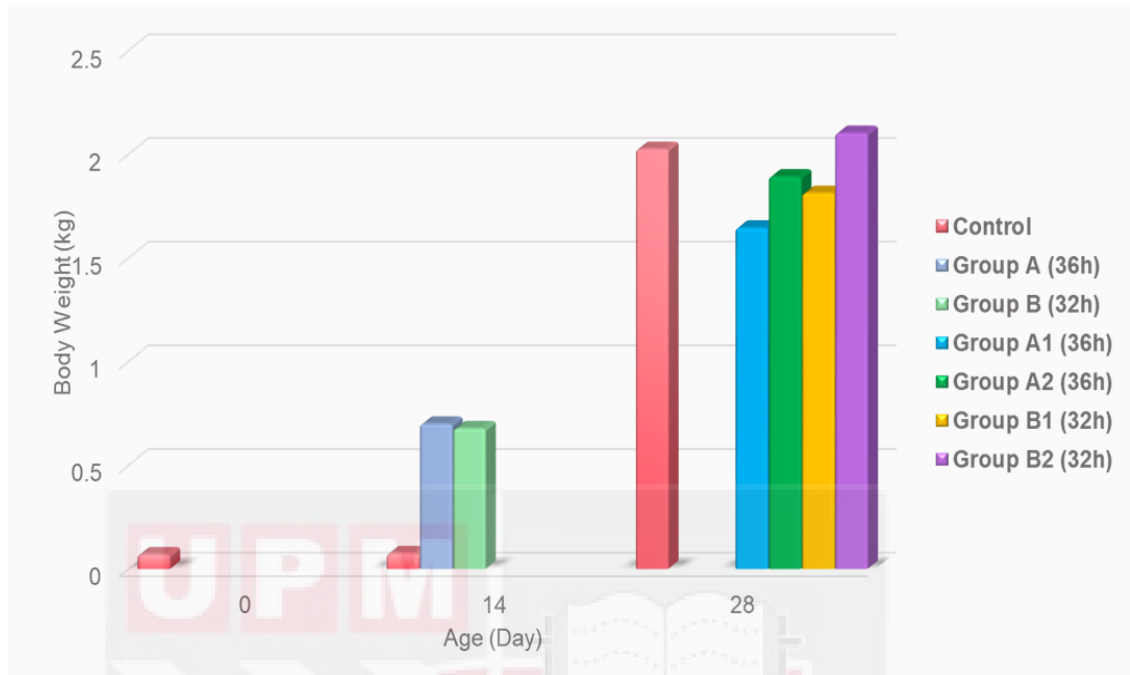
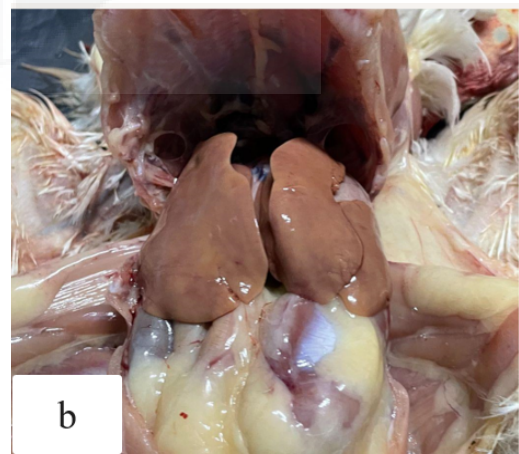


Figure 1: Body weight of chickens between inoculated (Group A and B) and control groups throughout the trial.

4.3 Gross Lesions

The liver of day old chicks were yellowish, while trachea were normal according to the chicks size. At day 14 and 28pi, all livers are dark and appear glistening in all groups with normal trachea. No gross lesion was observed in all liver and trachea samples in all groups at day 14 and 28pi (Figure 2).



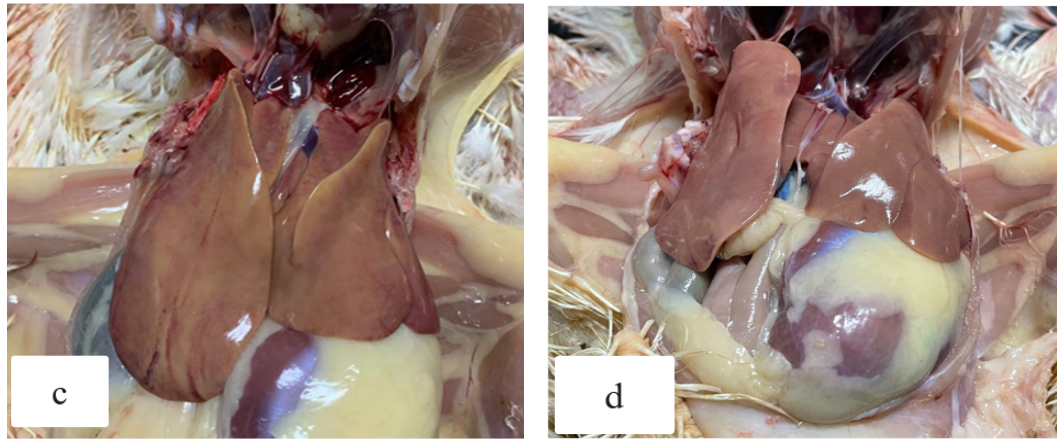
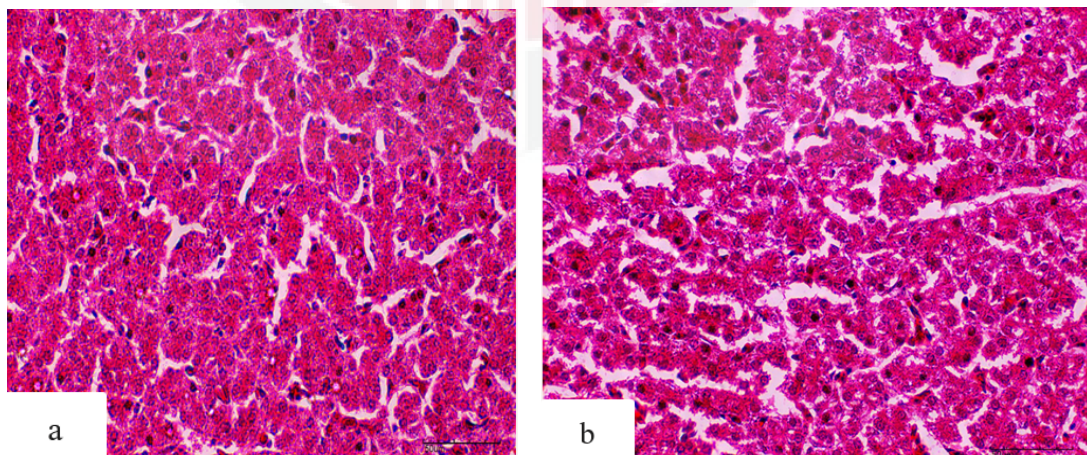


Figure 2: Necropsy finding of liver in chickens between booster and non-booster groups following vaccination with inactivated FAdV at 36h (group A) and 36h (group B) at day 28pi. Liver was normal with glistening surfaces from chicken groups (a): A1, (b): B1, (c): A2 and (d): B2.

4.4 Histopathological Lesions

There are no significant findings in both liver and trachea for all groups at day 0, 14 and 28pi. Livers were normal and retained original architecture without the presence of intranuclear inclusion bodies in the hepatocytes (Figure 3) Similarly, there is no presence of inflammatory cells in trachea tissue.



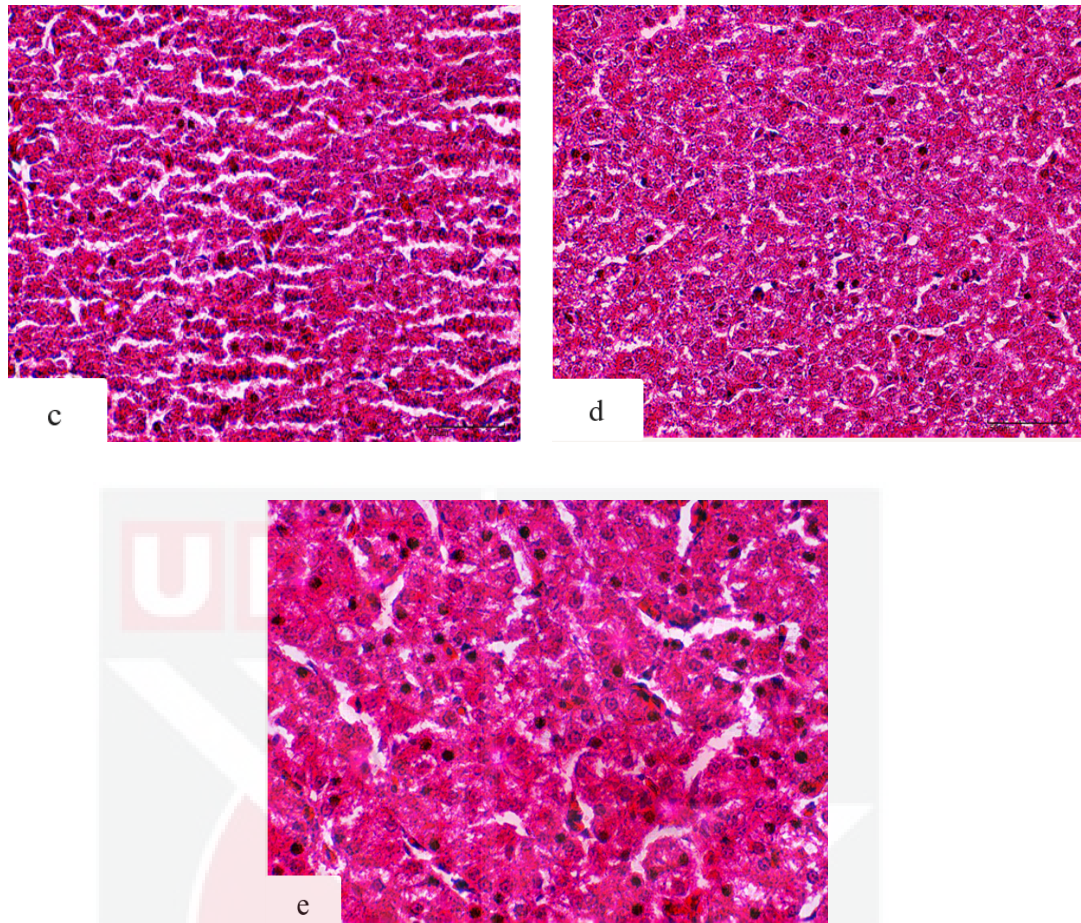


Figure 3: Histological finding of liver between inoculated (Group A and B) and non-inoculated (Control) groups at day 28pi. Normal finding of liver with normal architecture from groups (a): A1, (b): B1, (c): A2 and (d): B2 and (e): Control. HE, 40X. Bar= 20 μ m.

4.5 Antibody Titers

The FAdV antibody titer of day old chicks was 4469 ± 1175 (Figure 4)(**Appendix 3, Table A3**). The FAdV antibody titers for control groups for day 14 and 28 were 112 ± 106 and 147 ± 60 , respectively. At day 14pi, the antibody titers of the chicken in groups A and B were 78 ± 35 and 490 ± 360 , respectively which were not significantly different from each other ($p > 0.05$). At day 28pi, the antibody titers for groups control and non-booster inoculated groups, A1 and B1 were 1 ± 0 , 321 ± 189 ,

690 ± 484, respectively, in which the FAdV antibody titers of Group A1 and B1 were significantly higher ($p < 0.05$) compared to the control group. Similarly, antibody titers for booster groups A2 (602 ± 367) and B2 (874 ± 317) are significantly high ($p < 0.05$) compared to the control group. However, there is no significant difference ($p > 0.05$) on antibody titer between booster and non-booster groups regardless of type of time interval ($p > 0.05$).

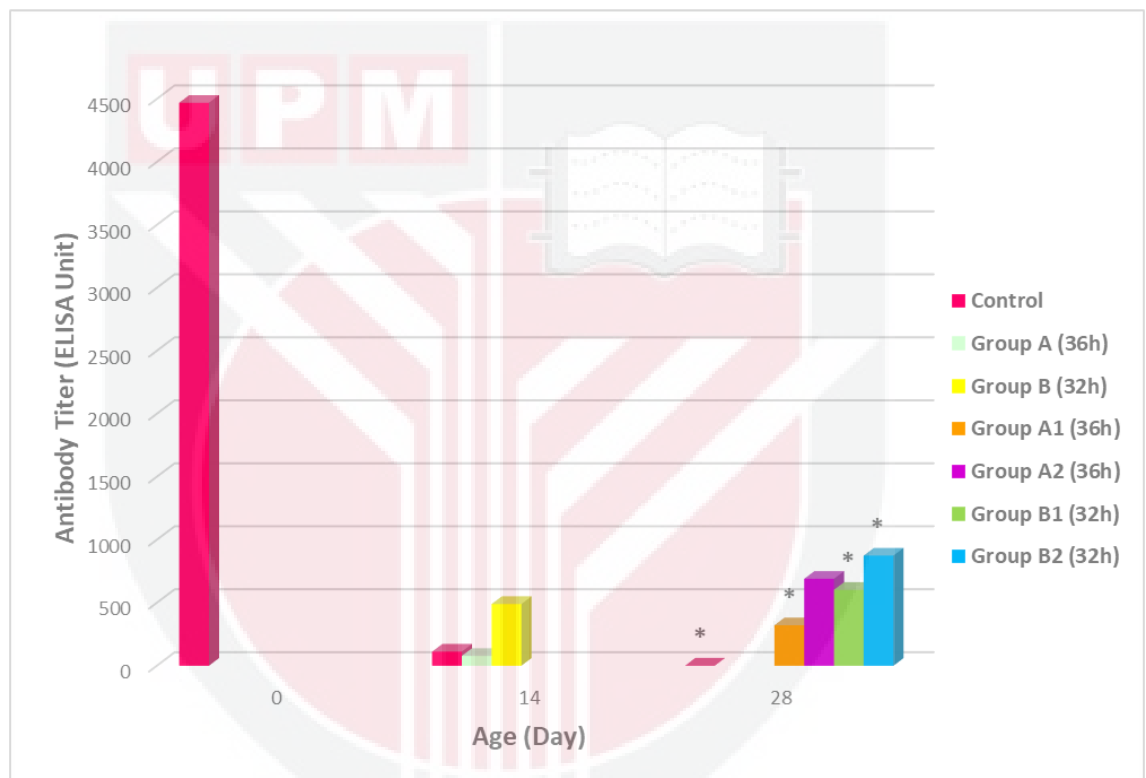


Figure 4: FAdV antibody titer of chickens between inoculated and control groups throughout the trial. FAdV antibody titers of inoculated groups, Group A1 and B1, were significantly higher ($p < 0.05$) compared to the control group at day 28pi. Similarly, antibody titers for booster groups A2 and B2 are significantly high ($p < 0.05$) compared to the control group. * Asterisk indicates a significant difference.

CHAPTER 5

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Efficacy of inactivated FAdV with different inactivation time interval was determined based on a few parameters which are the clinical signs, body weight, gross and histological lesions and mean antibody titer in five different groups of chicken (A1, A2, B1, B2 and C). In this study, there were no clinical signs of IBH and tracheitis shown and no mortality occurred in any commercial broiler chickens throughout the trial. Based on the days 28pi it was observed that chickens from all groups did not exhibit any clinical signs associated with inactivated FAdV inoculation that inactivated at different time intervals (32 and 36 hours).

There was no presence of gross and histological lesions based on necropsy findings in all groups throughout the trial. Gross lesions such as swollen, pale liver with hemorrhages and multifocal necrosis shown in most IBH cases were not found. No presence of lymphocytes infiltration that indicated typical tracheitis. In histological changes of liver, during IBH outbreaks, the infected chickens would produce eosinophilic or basophilic intranuclear inclusion bodies in the hepatocytes which is a typical characteristic of viral replication (Alemnesh *et al.*, 2012). According to a study conducted by Pan *et al* (2017), vaccinated chickens showed no obvious clinical signs or pathological lesions were observed in chickens in both single and double immunization groups which indicates the safety and the sufficient protection of the vaccine. Hence, this suggested that inactivated FAdV with different inactivation time intervals did not cause any gross or histological lesion on the liver and trachea

throughout the trial and there were no side-effects of the different inactivation time interval caused onto the chickens' grossly.

Based on this study, the body weight of chickens increased throughout 28 days of trial for all the groups. There was a significant difference ($p>0.05$) between groups in terms of the body weight on days 14 and 28 of all the commercial broiler chickens in different groups. The body weight of the chickens increased throughout the 28 days trial with all weights being more than 1.6kg. Based on the broiler performance objectives and guidelines from Cobb Vantress (2015), female and males Cobb 500 should be weighing 1.4kg and 1.6kg respectively on 28 days of age. Hence, this showed that the inoculation of inactivated FAdV inoculum did not affect the growth performance of broiler chicken.

Because the efficacy of the inactivated vaccine is related to an induction of the humoral immune response, the antibody level of vaccinated chickens could be a useful criterion for the evaluation of inactivated vaccines (Kim *et al.*, 2014). Based on the results of FAdV antibody titer, the chicks in the control group (C) had the highest FAdV antibody titer (9605 ± 3555) on day 0 pi but dropped significantly on day 14 pi (112 ± 106) and on the end of trail of day 28 pi (147 ± 60). The presence of high levels of FAdV antibodies in day-old chicks strongly suggested that breeding flocks may have been exposed to FAdV, thus passing maternal antibodies to the progenies. This phenomenon occurred because FAdV was ubiquitous and chickens were exposed to the field (McFerran and Smyth, 2000). This proves that the presence of maternal antibodies in an unvaccinated flock of broiler chicken suggests that there may be occurrence of field challenge in Malaysia poultry farms.

Another possibility is the breeder flock could have been vaccinated with FAdV strain previously which can be passed down to their progenies. This corresponds to a study by Toro *et al* (2002) where they mention that maternally derived antibodies to FAdV are known to protect chicks from infection with antibody-eliciting viruses. Because chicks develop an age-related resistance against FAdV (McFerran and Adair 2003), the vaccination of breeders to confer an adequate level of maternally derived antibodies in the progenies is effective in protecting young chicks from FAdV, prior to the development of age-related resistance (Kim *et al.*, 2014).

The FAdV antibody titer day 14 pi showed that Group B (490 ± 360) produced a higher antibody titer than Group A (78 ± 35). However, both groups showed variation in antibody response but there are not significantly different ($p>0.05$) between groups. This can be due to the small sample size and variation of chickens used in the trial. Therefore, inactivated FAdV vaccine with inactivated inoculum of 32 hours produced a significantly higher ($p<0.05$) FAdV antibody titer than inactivated FAdV vaccine with inactivated inoculum of 36 hours.

On day 28 pi, Group B2 produced the highest antibody titer compared to group control, group A1, group A2 and group B1. However, there is no significant difference between booster (A2 and B2) and non-booster (A1 and B1) groups regardless of the time interval of inoculum use. This suggested that the single inoculation or multiple inoculation of inactivated FAdV vaccine with different inactivation time intervals does not affect the antibody titer and both are safe and immunogenic in broiler chickens. To support this, based on previous study for vaccinated chickens, no obvious clinical signs and pathological damage were

observed in chickens of both the single and double immunization groups, suggesting the safety and sufficient protection of the vaccine (Pan *et al.*, 2017).

In terms of efficacy of vaccines, live attenuated vaccinations in poultry have a long process of virus attenuation. Incomplete attenuation of virus isolate can be dangerous and may induce disease in chicken. According to Dey *et al.*, (2019), there is a high risk of the attenuated strain reverting to virulence and become pathogenic in hens. Therefore, development of inactivated FAdV vaccine provides a better option to induce immunogenicity in chickens.

According to Delrue *et al* (2012), two major bases when it comes to inactivating a virus for a vaccine. First, the virus should be inactivated completely and have little to minor effect on viral antigenic properties. Second, viral epitopes important for induction of protective immunity should be preserved after inactivation. The immune system of the host has to recognize the neutralizing epitopes to produce neutralizing antibodies against the antigen. Virus inactivation and virus structure preservation is important when developing inactivated vaccines. Next, three principles that need to be addressed when the process of inactivation of the virus takes place. First, a quality control system to detect the safety of inactivated virus vaccines can be obtained by passaging the treated virus *in vitro* (Budowsky *et al.*, 1999). A quality control system can help in eliminating the possible virus aggregate formation, which can lead to virus escaping inactivation. Only when the vaccine passes such a test, the safety of the vaccine can be confirmed.

Next, it is crucial that during inactivation of virus to preserve the virus structure. This allows the preservation of viral entry-associated domains to induce a virus-neutralizing antibody response. Therefore, it is important to select inactivation

procedures that do not affect the viral structure. For instance, alkylating agents such as β -propiolactone (BPL), Binary ethylene imine (BEI) and radiation agents such as gamma irradiation and ultraviolet light which mainly act on the genome and most likely preserving the viral-neutralizing epitopes (Budowsky *et al.*, 1999). Unlike cross-linkers such as formaldehyde, glutaraldehyde, 2,2'-dithiodipyridine or denaturing procedures such as pH and temperature that act on viral proteins which modifies the viral-neutralizing epitopes which can lead to protein degradation (Delrue *et al.*, 2012). Furthermore, a quality control system needs to be establish to investigate the antigen quality. In this study, Enzyme-linked Immunosorbent assay (ELISA) is used for the determination of attachment of neutralizing antibodies to viral epitopes after inactivation.

Based on several studies, the best chosen method to inactivated FAdV isolate is by using binary ethylenimine (BEI) compared to formalin. Formalin-inactivated FAdV vaccines have been experimented in past studies and are underperformed when compared to BEI-inactivated FAdV vaccines. Studies performed by Mansoor *et al.*, (2011) and Ali *et al.* (2015) have shown that formalin-inactivated liver homogenate vaccines have several disadvantages including an unexpected immune response triggered by the vaccination, impurity and the possibility of other virus and bacteria being present in the vaccine.

According to Akhtar *et al* (2000) formalin-inactivated FAdV vaccine performed less in inducing high antibody titer compared to BEI-inactivated FAdV vaccine. Formalin react with many proteins which causes what is known as “membrane effect”. The membrane effect causes alterations to the surface protein of the virus. This reduces the antigenicity of an antigen which can introduce the disease into vaccinated flocks.

In this study, BEI has been used for development of inactivated FAdV vaccines because it is more stable and an alkylating agent that reacts very little to the proteins. Thus, not altering the antigenic components of the virus (Akhtar *et al.*, 2000; Kim *et al.*, 2014; Junnu *et al.*, 2015). Next, BEI acts on the genome which preserves the viral neutralizing epitopes for the induction of humoral immunity (Delrue *et al.*, 2012). According to Bahnemann (1990), BEI reacts with viral nucleic acids while preserving conformation and accessibility of epitope to a much greater extent than formalin and betapropiolactone (BPL).

Therefore, the best method to inactivate a virus for a vaccine development are the methods that mainly affect the genome which are alkylating agents such as BPL and BEI. BEI has two protonizable amino groups (Delrue *et al.*, 2012) If low concentration of BEI is used, the viral capsid is not alkylated but BEI passes through the capsid and alkylates the genome. N7-guanine of the genome acts as a nucleophile and reacts with electrophile BEI. Adenine alkylated via ring-opening reaction with BEI. Ring-opening reaction in RNA nucleotides is 2-3 times faster than DNA nucleosides. No reports of BEI interacting with proteins. Thus neutralizing viral epitopes are preserved. The preservation of viral structure is maintained if BEI is used in low concentration which preserves the viral-entry associated domains inducing a protective immune response (Delrue *et al.*, 2012).

Upon receiving a lethal dose of the novel FAdV- 4, full protections were provided in chickens immunized with the vaccine once or twice. Cytokines IL-4 (Th2 response) and IFN- γ (Th1 response) in the serum of chickens were detected 7 and 14 days after the first and second immunization by ELISA. Although single immunization is capable of providing a hundred percent protection while saving the vaccine cost and extra work, double immunization was recommended because of the higher antibody

responses and secretion of Th2 cytokine IL-4 (Pan *et al.*, 2017). In this study, the booster group was able to induce higher antibody titer than non-booster vaccinated chickens. FAdV booster vaccines have been suggested to induce memory CTL immunity. The study conducted by Gupta *et al.* (2017) had higher levels of cytotoxic CD8+ lymphocytes following booster vaccination.

Inactivation time plays a major role in completely inactivating a virus to induce immunogenicity in chickens. According to a study conducted by Junnu *et al* (2015), BEI treated FadV supernatant solutions were incubated at 37°C and were collected at 0, 6, 12, 24, 48 and 72 hours. Then, the treated supernatants were tested for pH and virus infectivity. The inactivated FAdV isolates were tested for virus infectivity by inoculation on monolayer chicken embryo liver (CEL) cells. The cytopathic effect of the inoculated CEL were daily observed for 5-7 days. It is concluded that 0.01M BEI at 37°C for 72h was the optimal condition for FAdV inactivation. If compared to this study, the concentration of BEI in this experiment is 0.002M inactivated at 32h under 37 °C already provided complete inactivation of FAdV. We can speculate that the concentration of BEI does correspondingly affect the inactivation time interval of virus to be completely inactivated without inducing infection.

Next, the type of virus does affect the inactivation time interval. Viruses are composed of two main components which are the viral genome (RNA or DNA) and the virus-coded protein capsid that surrounds the genome (Gelderblom., 1996). Both enveloped and non-enveloped viruses have these two. However, enveloped viruses have lipid bilayer membranes on the outer part of the virus. Non-enveloped virus do not have lipid bilayer membranes because they cause host cell lysis, cell lysis is the most common exit mode from the host cell. The virus will violate the integrity of the

cell membrane causing cell death and significant tissue damage to the host (Gelderblom., 1996). For enveloped virus have lipid membrane bilayer , during virus assembly and exit of host cells, the virus uses the cell membrane of the host to assemble their membrane known as envelope. This avoids the process of cell lysis and helps envelope virus escape the host immune system (Más & Melero, 2013).

FAdV is an icosahedral non-enveloped double stranded DNA virus (Hess, 2000).

Viruses such as Avian Coronavirus which causes Infectious Bronchitis in poultry or is an enveloped positive-stranded RNA virus (Cavanagh and Gelb, 2008).

Non-enveloped viruses lack a lipid bilayer membrane and enveloped viruses have glycoproteins which are the major type of protein present on the exterior of the membrane. The presence of a lipid envelope provides an operational method that separates these viruses into two distinct classes (MacLachlan and Duobvi, 2016, p. 8).

BEI is a photoactive hydrophobic alkylating compound, can inactivate enveloped viruses by covalently binding to the hydrophobic domains of the viral proteins present in the envelope lipid bilayer (Brown *et al.*, 1998). This makes the enveloped virus no longer infectious because the virus loses its fusion proteins on the enveloped lipid bilayer and cannot bind to receptor molecules losing its ability to infect. Thus, the enveloped virus is easier and faster to inactivate. BEI inactivates non-enveloped viruses by inactivating the viral RNA/DNA genome. Ethylenimine is the chemical reagent in the aziridine group of BEI that is active at the site of nucleic acid on viral RNA/DNA but does not react with other proteins that are present in the virus (Brown *et al.*, 1998). This indirectly makes the non-enveloped virus more difficult to inactivate due to its specificity and hence a longer time to inactivate. Comparison has

been made between different studies of viruses using inactivation by BEI. According to study by Jariyasantitham *et al* (2017), the inactivation of enveloped Avian Coronavirus takes 24 hours to completely inactivate by BEI. If it were compared to non-enveloped Fowl Adenovirus, it takes 32 hours to be completely inactivated by BEI. Therefore, the structure of viruses does play a major role in inactivation.

Based on Budowsky *et al* (1999), changes of the inactivation conditions will cause alterations to higher structure of viral components such as proteins and nucleic acid and indirectly may affect the rate of component reactions with ethyleneimine (EI). Therefore, optimization of inactivation such as minimum time interval for treatment, concentration of EI, ionic strength, temperature and pH would reduce the extent of side reactions affecting immunogenicity and specificity of killed vaccine.

It is clear that the level of IgG antibody is an important indicator to evaluate the effect of the vaccine. According to Zhao *et al* (2020), inactivated vaccines are able to induced humoral immunity effectively and efficiently. Because the efficacy of inactivated vaccines is related with induction of humoral immune response, antibody level of chicken is a significant criterion in evaluating inactivated vaccines (Kim *et al.*, 2014). According to the study, inactivated vaccine BEI group induced higher IgG levels compared to other groups indicating that BEI was able to elicit better humoral immune response. According to study by Pan *et al* (2017), inactivated FAdV vaccine elicited high levels of antigen-specific antibodies and TH2 cytokine IL-4. Th cell are differentiated into Th1 and Th2 lymphocytes subsets, Th 1 cells play important role in regulating cellular immune response and mostly influence by IFN- γ while Th2 cells activated the humoral and mucosal immunities that mainly controlled by IL-4 (Zhao *et al.*, 2020).

5.2 Conclusion

It was concluded that the inactivated FAdV vaccine with different inactivation time intervals did not result in any interference of growth performance which is body weight and also did not inflict any gross or histological lesions on the chickens' liver and trachea throughout the 28 days trial. The optimum period of FAdV inactivation was 32 hr based on the antibody response.

5.3 Recommendations

Efficacy of inactivated FAdV UPM1137 should be tested by challenge trial with pathogenic field FAdV isolate. It is important to evaluate the protection rate in the vaccinated chickens against the IBH and gizzard erosion. Next, it is recommended that the study of cross-protection of other FAdV serotypes should be considered. Lastly, other routes of administration of inactivated FAdV vaccine should be considered.

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APPENDIX 1

Table A1: Experimental design for safety and immunogenicity study of inactivated FAdV UPM1137

Groups	Time (Days post-inoculation)		
	0	14	28
A1 (Non-booster)	-	5	5
A2 (Booster)	-	-	5
B1 (Non-booster)	-	5	5
B2 (Booster)	-	-	5
Control	5	5	5
Total number of chicks	45		

Group A1: Chicken inoculated with vaccine using FAdV inactivated isolate at 36h and non-booster;

Group A2: Chicken inoculated with vaccine using FAdV inactivated isolate at 36h and booster;

Group B1: Chicken inoculated with vaccine using FAdV inactivated isolate at 32h and non-booster;

Group B2: Chicken inoculated with vaccine using FAdV inactivated isolate at 32h and booster;

Group C: Control.

APPENDIX 2

Table A2: Mean body weight of chickens throughout the trial.

Days post-inoculation (pi)	Group				
	Control	Group A1	Group A2	Group B1	Group B2
0	0.074±0.003 ^a				
14	0.753±0.017 ^b	0.7±0.027 ^b	-	0.678±0.022 ^b	-
28	1.888±0.101 ^c	1.646±0.165 ^c	1.893±0.061 ^c	1.813±0.06 ^c	2.103±0.027 ^c

Each value is the mean ± standard error mean of the 5 chickens from each group.

^{a-b-c} means within row with no common superscripts differs at $p < 0.05$.

APPENDIX 3

Table A3: Mean FAdV antibody titer of chickens throughout the trial

Days post-inoculation (pi)	Group				
	Control	Group A1	Group A2	Group B1	Group B2
0	4469 ± 1175 ^a				
14	112 ± 106 ^b	78 ± 35 ^b	-	490 ± 360 ^b	-
28	1 ± 0 ^c	321 ± 189 ^d	690 ± 484 ^d	602 ± 367 ^d	874 ± 317 ^d

Each value is the mean ± standard error mean of the 5 chickens from each group.

^{a-b-c-d} means within row with no common superscripts differs at $p < 0.05$.