UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY OF FOWLADENOVIRUS ISOLATES OF MALAYSIA IN SPECIFIC PATHOGEN FREE EMBRYONATED CHICKEN EGGS

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BY

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A project paper submitted to
the Faculty of Veterinary Medicine
Universiti Putra Malaysia
in partial fulfillment of the requirement for the
Degree of Doctor of Veterinary Medicine

FACULTY OF VETERINARY MEDICINE
UNIVERSITI PUTRA MALAYSIA
SERDANG
SELANGOR DARUL EHSAN

MARCH 2015

It is hereby certified that I have read this project paper entitled "Pathogenicity of Fowl Adenovirus Isolates of Malaysia in Specific Pathogen Free Embryonated Chicken Eggs" by Nurul Kamaliah binti Mustafa Kamal and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999-Project.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor Professor Dr. Mohd Hair Bejo for his guidance, encouragements, comments and suggestions throughout this study and preparation of this project paper.

I would also like thank the staffs of Serology Laboratory especially Dr. Norfitriah and Mrs. Mardhiah for their dedicated assistance, not forgetting Mr. Saipuzaman for his help and technical assistance.

To my beloved best friend especially Siti Nur Afiqah for happiness and joyfulness. I will remember forever our silly jokes until we can laugh together. I really enjoy and appreciate this friendship.

Not forgetting to my parents and siblings, my big family members at Ampang Jaya and Sungai Petani, those are always be with me during happy and sorrow. Special thank to my academic advisor, Dr. Siti Zubaidah Ramanoon, for her support from the first year of DVM until now.

Last but not least, Alhamdulillah, thank to Allah S.W.T, I am proud to be myself, as a woman who is enjoy to do everything in this world and never give up to reach the target goal.

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LIST OF ABBREVIATIONS

FAdV	Fowl Adenovirus
AAV	Avian Adenoviruses
^{o}C	Degree Celcius
HE	Hematoxylin and eosin
IBH	Inclusion body hepatitis
IBD	Infectious bursal disease
CAV	Chicken anaemia virus
CAM	Chorioallantoic membrane
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
SPF	Specific pathogen free
Pi	Post inoculation

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

PATHOGENESITI ADENOVIRUS AVIAN PENCILAN MALAYSIA DALAM TELUR AYAM BEREMBRIO BEBAS PATOGEN KHUSUS

Oleh

Nurul Kamaliah binti Mustafa Kamal

Mac 2015

Penyelia: Professor Dr. Mohd Hair Bin Bejo

Adenovirus avian (FAdV) adalah patogen utama inklusi badan hepatitis (IBH) dalam ayam dan menyebabkan kadar kematian yang tinggi. Objektif kajian ini adalah untuk menentukan patogenisiti FAdV pencilan Malaysia yang terkini dalam telur ayam berembrio bebas patogen khusus (SPF). Sebanyak tiga puluh sembilan SPF telur ayam berembrio berumur 8 hari telah dibahagikan kepada tiga kumpulan utama iaitu kumpulan A , B dan C. Dua belas telur daripada setiap kumpulan A dan B telah disuntik dengan 0.1mL/telur pencilan FAdV A (UPM11134) dan B (UPM1127) menerusi membran korioalantoik (CAM). Lima belas telur lagi daripada kumpulan C telah dibiarkan tanpa inokulasi dan digunakan sebagai kumpulan kawalan. Tiga biji telur

daripada kumpulan kawalan telah dikorbankan sebelum suntikan FAdV dijalankan. Kesemua telur diperiksa dua kali sehari dan kematian dicatatkan sepanjang 14 hari kajian. Sampel hati, hempedal dan CAM daripada embrio yang mati telah diawetkan di dalam 10% bufer formalin untuk pemeriksaan histologi. Kajian menunjukkan bahawa 100% kematian telah dicatatkan bagi kedua-dua kumpulan A dan B dalam masa 3 hingga 8 hari selepas inokulasi (pi). Walaubagaimanapun, kumpulan kawalan kekal dengan tiada kematian sepanjang kajian dijalankan. Lesi matakasar bagi kedua-dua kumpulan berlaku terutamanya di dalam CAM, hati dan hempedal. CAM yang tebal dan berwarna kelam diperhatikan bermula pada hari ke-3 pi. Lesi di hati telah diperhatikan bermula pada hari ke-3 pi dan ia menunjukkan pembengkakan dan pendarahan. Hidroperikardium beserta dengan nekrosis di pelbagai kawasan hati dicatatkan bermula hari ke-7 pi. Manakala hempedal pula kekal normal bagi kedua-dua kumpulan. Histologi menunjukkan kehadiran beberapa jasad rangkuman intranuklear (INIB) di CAM, hati dan hempedal untuk kumpulan A dan B. Walaubagaimanapun, kumpulan kawalan kekal normal sepanjang eksperimen dijalankan. Disimpulkan bahawa kedua-dua pencilan FAdV Malaysia adalah sangat patogenik kepada SPF telur ayam berembrio dan hati embrio harus digunakan untuk pengasingan dan penyebaran virus.

Kata kunci: Adenovirus Avian (FAdV), telur ayam berembrio bebas patogen khusus (SPF), patogenisiti, membran korioalantoik (CAM), jasad rangkuman intranuklear (INIB).

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 - Project.

PATHOGENICITY OF FOWL ADENOVIRUS ISOLATES OF MALAYSIA IN SPECIFIC PATHOGEN FREE EMBRYONATED CHICKEN EGGS

by

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2015

Supervisor: Professor Dr. Mohd Hair Bin Bejo

Fowl Adenovirus (FAdV) is known to be the primary pathogen of inclusion body hepatitis (IBH) and can cause high mortality in susceptible chickens. It was the objective of this study to determine the pathogenicity of recent FAdV isolates of Malaysia in specific pathogen free (SPF) embryonated chicken eggs. Thirty nine 8-day-old SPF embryonated chicken eggs were divided into three groups namely groups A, B and C. Twelve eggs from groups A and B were inoculated with 0.1mL/egg of FAdV isolates A (UPM11134) and B (UPM1127), respectively via chorioallantoic membrane (CAM) route. Fifteen eggs from Group C were left uninoculated and used as the control group. Three eggs from the control group were sacrificed prior to FAdV inoculation. All eggs

were candled twice daily and any mortality was recorded throughout 14 days trial. Samples of liver, gizzard and CAM of the dead embryos were fixed in 10% buffered formalin for histological examination. The study showed that both FAdV isolates caused 100% mortality when inoculated in 8-day-old SPF eggs within 3 to 8 days post inoculation (pi). The control group remained no mortality throughout the trial. The gross lesions recorded were mainly in CAM, liver and gizzard. The CAM was thickened and cloudy as early as day 3 pi. Lesions in liver revealed enlarged and ecchymotic haemorrhage was first observed at day 3 pi. The lesion became severe with the presence of hydropericardium and multifocal areas of necrosis in liver on day 7 pi. The gizzard was normal for both groups. Histologically, basophilic intranuclear inclusion bodies were observed in CAM, liver and gizzard in groups A and B. The control group remained normal throughout the trial. It was concluded that the FAdV isolates of Malaysia is highly pathogenic to SPF embryonated chicken eggs and the embryonic liver should be used for isolation and propagation of the virus.

Keywords: Fowl Adenovirus (FAdV), specific pathogen free (SPF) embryonated chicken eggs, pathogenicity, chorioallantoic membrane (CAM), intranuclear inclusion body (INIB).

INTRODUCTION

Fowl Adenoviruses (FAdVs) belong to the family *Adenoviridae* and classified in the genus *Aviadenovirus.* FAdVs were grouped into five different species (A to E) and they are further subdivided into 12 serotypes from chickens, geese, ducks, turkeys and pigeons (Mase *et al.,* 2009 ; Philippe *et al.,* 2005). FAdVs are mainly responsible for naturally acquired outbreaks of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), respiratory disease and gizzard erosions (Adair and Fitzgerald, 2008).

IBH was first described in the USA in 1963 (Helmboldt and Frazier, 1963) and then this disease was rapidly spread all over the world. IBH has emerged as an economically important disease in broiler chickens recently and outbreaks of the disease were reported worldwide including USA, Canada, England, Germany, Pakistan, India, Korea, Japan, Australia and New Zealand (Alvarado *et al.,* 2007 ; Christensen and Saifuddin, 1989 ; El-Attrache and Villegas, 2001 ; Gomis *et al.,* 2006 ; Nakamura *et al.,* 2011 ; Ojkic *et al.,* 2008 ; Steer *et al.,* 2011). The serotype causing IBH is differ according to geograhic regions. In Canada, Australia and New Zealand, FAdV-8 has been isolated from most of the outbreaks in the commercial broiler chickens (Kefford e*t al.,* 1980 ; Ojkic *et al.,* 2008 ; Saifuddin and Wilks, 1990 ; Saifuddin *et al.,* 1992). IBH cases become epidemic recently in Japan and it is associated with FAdV-2 (Nakamura *et al.,* 2011). In Malaysia, the first report of the disease was in 2005 (Hair-Bejo, 2005). Recently, five FAdV

isolates of Malaysia involved in IBH outbreaks have been successfully propagated in SPF embryonated chicken eggs n they are characterized as FAdV-8b (Juliana *et al.,* 2014).

IBH is usually seen in broiler chickens aged 3 to 7 weeks. It is characterized by a sudden increase in mortality which ranges from 2 to 10%. The varying degree of mortality correlates to the pathogenicity of the virus, secondary infections and susceptibility of the chickens (Grgic *et al.,* 2011). Generally, the mortality peaked on day 3 and remained high through day 5 post infection. The majority of clinical signs and mortality stopped by day 7 after infection (Ritchie, 1995). However, the morbidity is low. Sick birds adopt a crouching position with ruffled feathers and died within 48 hours or recovered. However, it must be stressed that infections with FAdV occur frequently in healthy birds. The other birds in the flock often appear normal or they may be depressed for a few days (Smyth and McNulty, 2008). The main lesions of IBH are pale, friable and swollen liver. Petechial or ecchymotic haemorrhages may be present in the liver and skeletal muscles. Intranuclear inclusion bodies are seen in the hepatocytes microscopically. These can be either eosinophilic or basophilic. Virus particles were detected only in cells with basophilic inclusions, and the eosinophilic inclusions were composed of a fibrillar, granular material and filaments (Adair and Fitzgerald, 2008).

Transmission of IBH occurs both horizontally and vertically. Horizontal spread within flock seemed to take place mainly by direct fecal contact but was also achieved

by aerosol over short distances, with infection spreading at a very slow rate for several weeks. In addition, fomites, personnel and transport can also be important for spreading of the agent as FAdV is relatively resistant to inactivation. Following primary infection or reactivation of latent virus, the virus can be transmitted vertically through embryonated egg as this become the main method of spread from one generation of intensively reared poultry to the next generation. FAdV is believed to act as a secondary pathogen, following infection with an immunosuppressive agent, such as infectious bursal disease virus (IBDV) or chicken infectious anaemia virus (CAV). However, FAdV has been reported as the primary pathogen of IBH (Philippe *et al.,* 2005).

FAdVs can be isolated from affected samples such as faeces, pharynx, trachea, liver and gizzard. In case of IBH, the specimen of choice for virus isolation is the liver (Adair and Fitzgerald, 2008). Besides, isolation of FAdV can be performed on chicken embryo liver (CEL) cell culture, kidney cell culture or in the embryonated eggs (Marek *et al.,* 2010). CEL cells are preferable rather than kidney cells for diagnostic purposes because of their greater sensitivity to other viruses. The chorioallantoic membrane route of inoculation in embryonated eggs was found to be more sensitive for virus isolation than the allantoic cavity (Adair and Fitzgerald, 2008).

1.1 Objectives

The objective of this study was to determine the pathogenicity of two recent FAdV isolates of Malaysia in specific pathogen free (SPF) embryonated chicken eggs.

LITERATURE REVIEW

2.1 Adenovirus Infection in Poultry

2.1.1. Introduction

Adenoviruses are members of the family *Adenoviridae,* which currently comprise of five genera which are *Mastadenovirus, Aviadenovirus, Siadenovirus, Atadenovirus* and *Ichtadenovirus* (Harrach and Kájan, 2011). Adenoviruses that infect mammals belong to the genera *Mastadenovirus* and *Atadenovirus,* while those that infect birds belong to the three genera: *Aviadenovirus, Siadenovirus* and *Atadenovirus* (Smyth and McNulty, 2008). *Aviadenovirus* can be found from chickens, turkeys, geese, ducks and pigeons. Besides, *Siadenovirus* consists of the viruses causing haemorrhagic enteritis in turkeys, marble spleen disease in pheasants, and splenomegaly in chickens. *Atadenovirus* contains the virus isolated from ducks and chickens which is responsible for egg drop syndrome (Mase *et al.,* 2009). *Ichtadenovirus* is the newly establish genus which has been isolated from fish. It was originated from white sturgeon.

2.1.2. Fowl Adenovirus Infection

Fowl Adenovirus (FAdV) infection is a worldwide distribution and is mainly responsible for naturally acquired outbreaks of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), respiratory disease and gizzard erosions (Adair and Fitzgerald, 2008). Other avian species has FAdV infection such as quail and turkey

causing quail bronchitis and viral hepatitis, respectively (Smyth and McNulty, 2008).

2.2 Aetiology

2.2.1. Classification

FAdVs are grouped into five different species (A to E) based on their hexon gene sequence. These species are further differentiated into 12 serotypes (FAdV-1 to FAdV-8a, FAdV-8b, and FAdV-9 to FAdV-11) based on cross-neutralization test (Benko *et al.,* 2005; Hess *et al.,* 2000).

2.2.2. Morphology

Adenoviruses are icosahedral, non-enveloped double-stranded DNA viruses that range in size from 70 to 90 nm (Adair and Fitzgerald, 2008). They have 252 structural units or capsomeres, which surround a core of 60 to 65nm in diameter and are arranged into 20 triangular faces with 12 vertices. The capsomere found at the vertices are called pentons, while the non-vertex capsomeres are called hexons. Each triangular face has six capsomeres along each edge. One pin-shaped projection called fibre, projects from each vertex of members of the *Mastadenovirus, Siadenovirus* and *Atadenovirus* genera, while members of the *Aviadenovirus* genus have two fibres at each vertex (Smyth and McNulty, 2008).

2.2.3. Effects of Chemical and Physical Agents

All avian adenoviruses are resistant to lipid solvents such as ether and chloroform, sodium deoxycholate, trypsin, 2% phenol, and 50% alcohol. They are resistant to variations of pH between 3 and 9, but are inactivated by 1:1000 concentration of formaldehyde. Although it is accepted that adenoviruses in general are inactivated in aqueous solution after exposure to 56^{o}C for 30 minutes and the heat stability is reduced by divalent ions, avian adenoviruses show more variability and are apparently more heat resistant. Some strains survive at 60^{o}C and even 70^{o}C for 30 minutes. The infectivity titre of one FAdV-1 virus fell rapidly after 180 minutes at 56^{o}C, and another FAdV-1 strain apparently survived 18 hours at 56^{o}C (Smyth and McNulty, 2008).

2.3 Epizootiology

FAdVs are ubiquitous in domestic fowl and frequently of low virulence (Philippe *et al.,* 2005). Thus, the role of most FAdV isolates as pathogens for chickens has not well defined, as FAdV can be isolated from sick and clinically normal chickens (Adair and Fitzgerald, 2008 ; Philippe *et al.,* 2005). Mixed infections with different FAdV serotypes can occur in the same bird (Meulemans *et al.,* 2001). Transmission of IBH occurs both horizontally and vertically. Horizontal spread within flock seemed to take place mainly by direct fecal contact, but was also achieved by aerosol over short distances, with infection spreading at a very slow rate for several weeks (Smyth and McNulty, 2008). In addition, fomites, personnel and transport can also be important for spreading of the agent as FAdVs are relatively resistant to inactivation. Following primary infection or

reactivation of latent virus, FAdV can be transmitted vertically through the embryonated egg. This is the main method of spread from one generation of intensively reared poultry to the next generation. Vertical infections are controlled by maternally derived antibodies. Virus shedding in faeces increases as maternally derived antibodies decreases. FAdV is believed to act as a secondary pathogen, following infection with an immunosuppressive agent, such as infectious bursal disease virus (IBDV) or chicken infectious anaemia virus (CAV) (Philippe *et al.,* 2005 ; Choi *et al.,* 2012). However, FAdV has been reported as the primary pathogen of IBH (Philippe *et al.,* 2005).

2.4 Pathogenesis

Experimental infection of SPF chicks in the first few days of life indicates the virus replicate in the epithelium of both large and small intestines following inhalation or ingestion of virus (Smyth and McNulty, 2008 ; Russell, 2009). A viraemia then occurs, allowing virus to be detected in many organs including liver, kidney, respiratory tract, bursa of Fabricius, spleen and bone marrow (Smyth and McNulty, 2008). However in the field, infections with FAdV are not normally detected during the first few days of life, but isolation from 3 weeks onwards are common. Virus can be readily isolated from faeces, ocular and nasal mucosa, and bursa of Fabricius. In chickens, the incubation period following natural infection is considered to be 24 to 48 hours. FAdV is excreted in the faeces for about 3 weeks, with the peak excretion between 4 and 7 days after infection (Smyth and McNulty, 2008 ; Adair and Fitzgerald, 2008). Furthermore, FAdV can establish latent infections and the infected chickens are potential lifelong carriers.

Reactivation of latent virus causes a recall of serum antibody to the common group antigen and, if the birds are in lay, virus may be transmitted through the egg. Thus, FAdV is commonly isolated from hens around the time of peak egg production (Smyth and McNulty, 2008).

2.5 Clinical signs

Inclusion body hepatitis (IBH) is usually seen in broiler chicken aged 3-7 weeks. It is characterized by a sudden increase in mortality which usually ranges from 2 to 10%. The varying degree of mortality correlates to the pathogenicity of the virus, secondary infections and susceptibility of the chickens (Grgic *et al.,* 2011). Generally, the mortality peaked on day 3 and remained high through day 5. The majority of clinical signs and mortality stopped by day 7 after infection (Smyth and McNulty, 2008 ; Adair and Fitzgerald, 2008). Morbidity is low and the sick birds showing a crouching position with ruffled feathers and die within 48 hours or recover. (Adair and Fitzgerald, 2008). However, it must be stressed that FAdV infections occur frequently in healthy birds. The other birds in the flock often appear normal or they may be depressed for a few days (Smyth and McNulty, 2008).

2.6 Pathology

2.6.1 Gross Lesions

The main lesions of IBH are pale, friable and swollen liver. Petechial or ecchymotic haemorrhages may be present in the liver and skeletal muscles (Smyth and McNulty, 2008). Swollen, pale kidney, and pale bone marrow are usually present at necropsy (Philippe *et al.,* 2005).

2.6.2 Histological Lesions

Intranuclear inclusion bodies (INIBs) are often observed in the hepatocytes. These can be eosinophilic or basophilic. Virus particles were detected only in cells with basophilic inclusions which are large in size and filling the whole nucleus. On the other hand, eosinophilic inclusions were composed of a fibrillar and granular material (Smyth and McNulty, 2008 ; Hair-Bejo, 2005). These suggest that basophilic INIBs consist of adenovirus particles, while eosinophilic inclusions contain only fibrillar granular material and filaments, which may represent a degenerative process or an early stage in the formation of virus.

2.7 Diagnosis

Observation of the gross lesion as well as histopathological changes in liver are the classical diagnosis of IBH. Virus isolation in cell culture or embryonated eggs was commonly performed for futher typing of the virus (Marek *et al.,* 2010 ; Juliana *et al.,*

2014). The chorioallantoic membrane (CAM) route of inoculation in embryonated eggs was found to be more sensitive for virus isolation than the allantoic cavity (Adair and Fitzgerald, 2008). However, not all FAdV serotypes can adapt and cause recognizable lesions in embryonated eggs (Juliana *et al.,* 2014). As an alternative, most chicken isolates have been made in chick kidney (CK) or chicken embryo liver (CEL) cells. CEL cells are preferable for diagnostic purposes because of their greater sensitivity to other viruses. FAdV infections also can be diagnosed by electron microscopy for direct examination or immunohistochemistry due to the adenovirus characteristic morphology. It also has been utilized to observe inclusion bodies in intestinal epithelium and liver. In order to determine the specific serotype, micro neutralization methods has been widely used. In addition, PCR has been developed to identify all avian adenoviruses which is useful for virological routine diagnosis of all field avian adenoviruses (Hess, 2000 ; Mase *et al.,* 2009 ; Juliana *et al.,* 2014).

2.8 Prevention and Control

Adenoviruses are resistant to inactivation when outside the host, and can remain infectious for a long period in litter, food, water or contaminated faeces. Adenoviruses can be inactivated by treatment for more than one hour with formalin, aldehydes or iodophors. There is no specific therapy for most adenovirus infections. Supportive care that includes maintaining adequate hydration and broad-spectrum antibiotics to prevent secondary infections may reduce the level of mortality in affected birds (Ritchie, 1995). Vaccination of broilers with chicken embryo lethal orphan virus (CELO) were proved to

reduce the clinical signs following challenge with a virulent FAdV-1 which is responsible for gizzard erosion (Grafl *et al.,* 2014). Therefore, oral vaccination with an apathogenic FAdV-1 was demonstrated to be an effective prophylaxis to protect broilers from horizontally induced adenoviral gizzard erosion. However, vaccination does not prevent excretion of challenged virus, although the birds are protected from clinical disease, as the virulent FAdV-1 was detected in faecal sample at day 10 post infection (Grafl *et al.,* 2014). In Australia, commercial live vaccine containing FAdV-8b strain has been used to vaccinate the broiler breeders. This vaccines had been proven to be effectively control the vertical transmission of FAdV, subsequently prevents the IBH outbreaks (Steer *et al.,* 2011). Maternal antibody titres of 1/64 or greater were successful in preventing the development of IBH, but at the same time it cause retardation of growth (Adair and Fitzgerald, 2008).

MATERIALS AND METHODS

3.1 FAdV Isolates

Two different FAdV isolates were kindly prepared by Prof. Dr. Mohd Hair Bejo. The isolates were obtained from field outbreaks of IBH in broiler chicken farms. Briefly, the first isolate was originated from 24-day-old chickens and known as isolate A (UPM11134). Enlarged and pale, with the presence of multifocal areas of necrosis as well as mild haemorrhage were recorded in the liver of the affected chickens. Mortality of 50% was recorded in SPF embryonated chicken eggs within 10 days pi when the liver sample inoculums was inoculated into the eggs via CAM route. PCR result from the liver samples revealed positive reaction for FAdV (Juliana, 2014). The liver sample from the embryo was used as FAdV inoculum in the present study.

The second isolate was originated from 18-day-old broiler chickens and known as isolate B (UPM1127). Clinical signs reported were pale shanks and combs with poor body weight. The mortality of 0.5 to 1% per day was recorded started from day 7 of age. Pale, swollen and yellowish with multifocal areas of necrosis and haemorrhages were recorded in the liver of the affected chickens. Mortality of 60% was recorded in SPF embryonated chicken eggs within 10 days pi when the liver sample inoculums was inoculated into the eggs via CAM route. PCR result from the liver samples revealed positive reaction for FAdV (Juliana, 2014). The liver sample from the embryo was used as FAdV inoculum in the present study.

3.2 Experimental Design

3.2.1 Pathogenicity of Two Different FAdV Isolates

Thirty-nine SPF embryonated chicken eggs were divided into three groups namely, groups A , B and C. Twelve eggs from groups A and B were inoculated with 0.1mL/egg FAdV isolates A and B, respectively. Fifteen eggs from group C were left uninoculated and used as the control group (Appendix 1). The eggs were candled twice daily and embryo mortality was recorded throughout the trial. The gross lesions were recorded upon necropsy of the dead embryos and liver, gizzard and chorioallantoic membrane (CAM) samples were fixed in 10% buffered formalin for histological examination.

3.2.2 Egg Inoculation

Prior to virus inoculation, all eggs were candled for viability and the sites of inoculation were disinfected with a solution of 70% ethyl alcohol. The eggs was marked at the midway along the long axis where there was fewer veins present during candling. A small hole was then drilled through the egg shell at the center of the air sac, as well as on the marked site before. New false air sac was created by performing suction at the original air sac using rubber suction. The egg was candled again to confirm the dropping of the CAM. By using 1 mL syringe with 25-gauge, 0.1 mL inoculum was inoculated into each egg by injecting it into the false air sac. Both holes were sealed with paraffin wax and the eggs were kept back in incubator (Laver, 1971) (Appendix 2).

3.2.3 Gross Lesions

The gross lesions of the embryo and CAM during necropsy were recorded.

3.2.4 Histological Lesions

Liver, gizzard and CAM were collected from embryonated eggs for histological examination. These tissues were fixed in 10% buffered formalin for at least 24 hours, then trimmed, subsequently dehydrated in series of alcohol, cleared with xylene and embedded in paraffin wax using an automatic tissue processor (Leica). The tissues were sectioned at 4 micrometer with microtome and mounted on glass slides, followed by dewaxing and finally stained with HE. The tissues were then carefully examined under a microscope and all pathological changes were recorded (Toro *et al.,* 2002).

RESULTS

4.1 Mortality

4.1.1 Group A

Eight out of 12 embryos died at day 3 pi, followed by another 2 embryos at day 4 pi. Subsequently, each embryo died at days 6 and 8 pi. Group A achieved 100% mortality at day 8 pi. The percentage of cumulative mortality for Group A was 67%, 83%, 92% and 100% at days 3, 4, 6 and 8 pi, respectively (Figure 1 and Apprendix 3).

4.1.2 Group B

Three embryos died at day 3 pi followed by 5 embryos at day 4 pi. At day 6 pi, an embryo was died and another 3 embryos died at day 7 pi. Group B achieved 100% mortality at day 7 pi, a day earlier compared to Group A. The percentage of cumulative mortality for group B was 25%, 67%, 75% and 100% at days 3, 4, 6 and 7 pi, respectively (Figure 1 and Apprendix 3).

4.1.3 Group C

No mortality was recorded throughout the trial period.

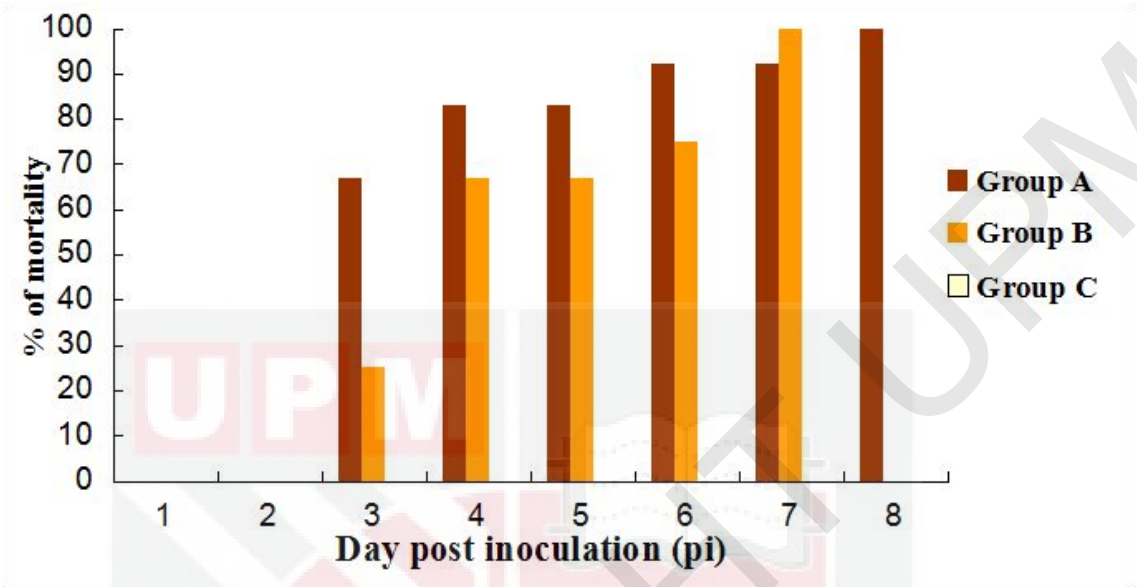

Figure 1: Cumulative mortality of the embryonated chicken eggs in groups A, B and C throughout the trial.

4.2 Gross Lesions

4.2.1 Group A

At day 3 and 4 pi, the embryos were congested with pale and enlarged liver (Figure 2a). The CAM revealed thickened and cloudy. The embryo was also congested at day 6 pi, with enlarged and congested liver and kidney. CAM was remained thickened and cloudy. During day 8 pi, the embryo was congested. The liver was markedly enlarged and pale with multifocal areas of necrosis (Figure 2b). The CAM was markedly thickened and cloudy (Figure 3a). The gizzard looks normal grossly throughout the trial.

4.2.2 Group B

Congested embryos with ecchymotic haemorrhage of the liver were recorded at day 3 pi (Figure 3b). There is also the presence of muscle oedema and swollen kidney. The CAM was thickened and cloudy. The embryos were remain congested at days 4 and 6 pi. The liver and kidney were enlarged. The CAM remained thickened and cloudy. Other than congested embryo, kidney and liver at day 7 pi, the liver revealed multifocal area of necrosis (Figure 3c). In addition, urate deposition in the kidney was observed (Figure 4a). Hydropericardium also was noticed with the presence of straw-coloured fluid in the pericardium (Figure 4b). The CAM was markedly thickened and cloudy. The gizzard revealed normal grossly throughout the trial period.

4.2.3 Group C

The embryos in the Group C remained normal throughout the trial period.

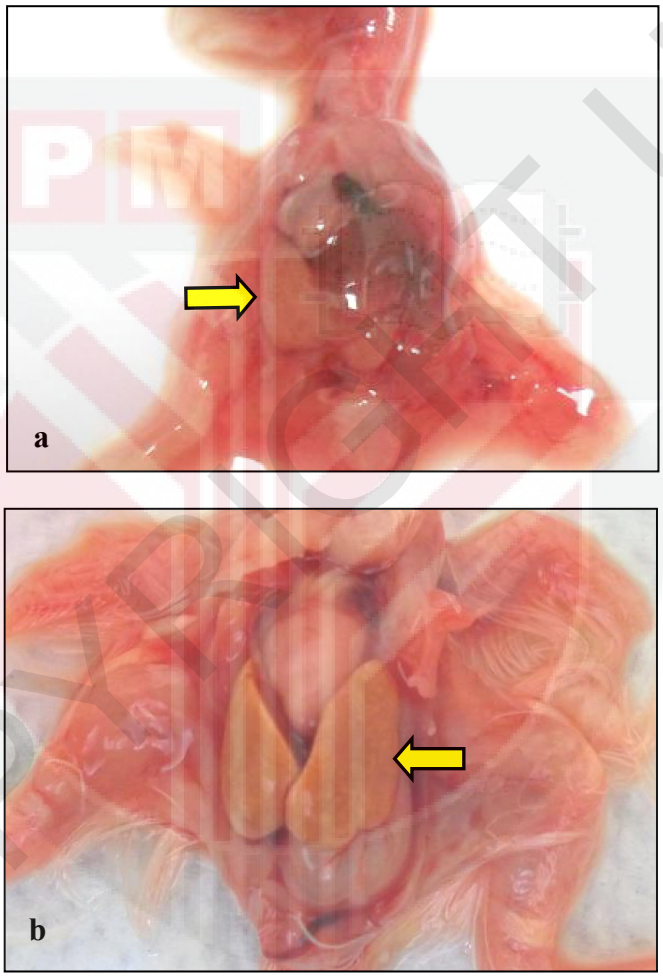

Figure 2: Group A. (a) Pale and enlarged liver at day 3 pi, and (b) liver was markedly enlarged and pale with multifocal areas of necrosis at day 8 pi.

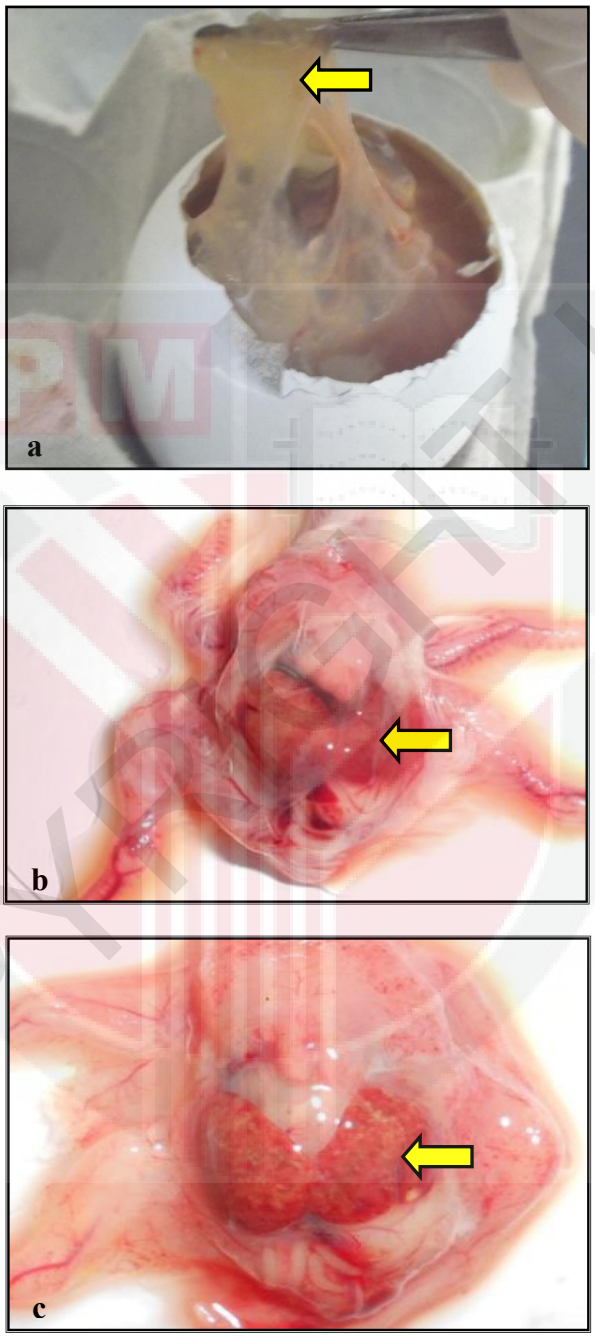

Figure 3: (a) Group A. Markedly thickened and cloudy CAM at day 8 pi. Group B.(b) Congested embryo with ecchymotic haemorrhage of the liver at day 3 pi, and (c) enlarged and congested liver with multifocal areas of necrosis at day 7 pi.

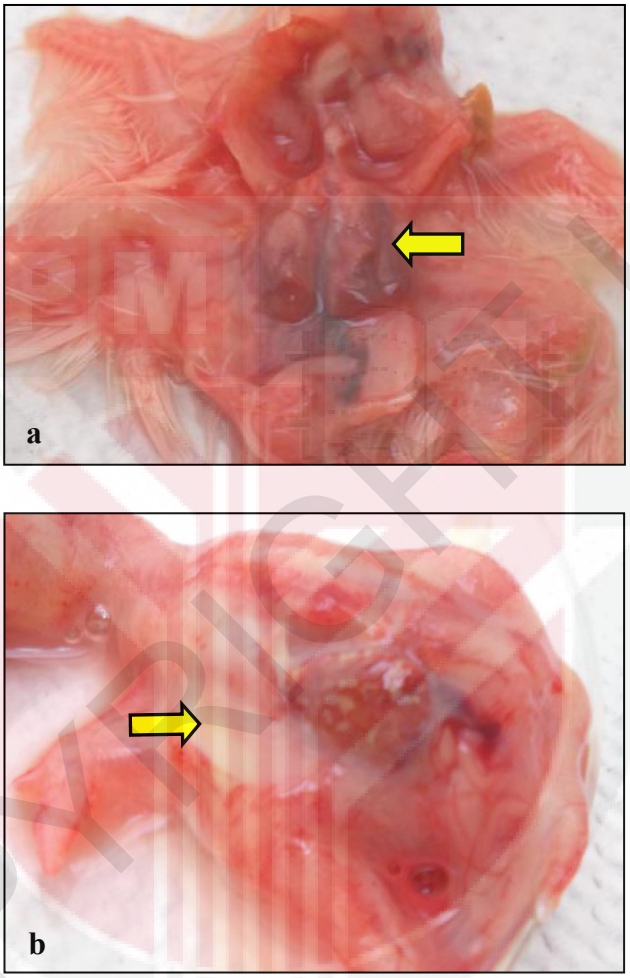

Figure 4: Group B. (a) Enlarged and congested kidney with urate deposition, and (b) hydropericardium at day 7 pi.

4.3 Histopathology

4.3.1 Group A

Liver samples

At days 3, 4 and 6 pi, mild to moderate haemorrhage, degenaration and necrosis of the hepatocytes with few basophilic intranuclear inclusion bodies were recorded (Figure 5a). Numerous inflammatory cells infiltration was also observed. There were also presence of multiple vacuolation in the cytoplasm of hepatocytes. Severe haemorrhage was recorded at day 8 pi. There was also presence of severe degenaration and necrosis with extensive infiltration of inflammatory cells and numerous large basophilic intranuclear inclusion bodies in the hepatocytes (Figure 5b).

Gizzard samples

At days 3 and 4 pi, numerous infiltration of inflammatory cells was recorded with a few basophilic intranuclear inclusion bodies in the epithelial cells. Numerous large basophilic intranuclear inclusion bodies were also observed at days 6 and 8 pi (Figure 6a).

Chorio-allantoic membrane (CAM) samples

A few basophilic intranuclear inclusion bodies were recorded in the epithelial cells at days 3 and 4 pi. Numerous large basophilic intranuclear inclusion bodies were also observed at day 6 pi (Figure 6b). Hyperplasia of the chorionic epithelium with large

basophilic intranuclear inclusion bodies was observed at days 8 pi.

4.3.2 Group B

Liver samples

At days 3 and 4 pi, mild to moderate degenaration and necrosis of the hepatocytes with congestion of the sinusoids was recorded. A few basophilic intanuclear inclusion bodies were observed (Figure 7a). At days 6 and 7 pi, moderate to severe congestion, haemorrhage, and extensive degeneration and necrosis with numerous basophilic intanuclear inclusion bodies were observed (Figure 7b).

Gizzard samples

Numerous infiltration of inflammatory cells was recorded with a few basophilic intranuclear inclusion bodies were observed in the epithelial cells at days 3, 4, 6 and 7 pi (Figure 8).

Chorio-allantoic membrane (CAM) samples

At days 3 and 4 pi, a few basophilic intranuclear inclusion bodies were recorded (Figure 9a). Hyperplasia of chorionic epithelium with numerous large basophilic intranuclear inclusion bodies were recorded at days 6 and 7 pi (Figure 9b).

4.3.3 Group C

All the samples remained normal throughout the trial period.

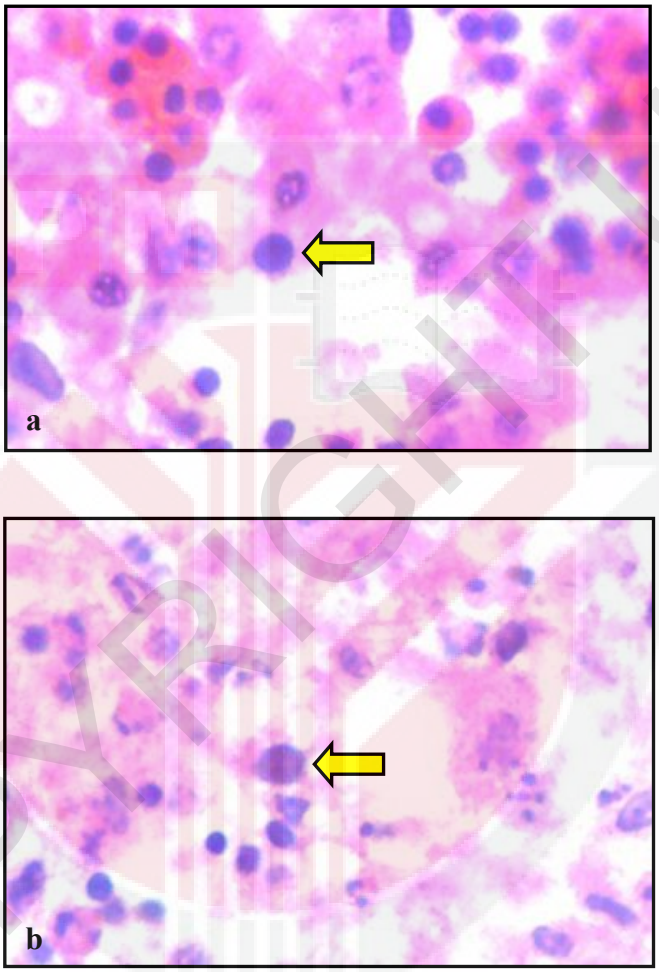

Figure 5: Group A. (a) Mild haemorrhage, degenaration and necrosis of the hepatocytes with the presence of basophilic intranuclear inclusion body in hepatocytes of embryo at day 3 pi, and (b) intranuclear inclusion body in hepatocyte of embryo with extensive degenaration and necrosis at day 8 pi. HE, 1000x.

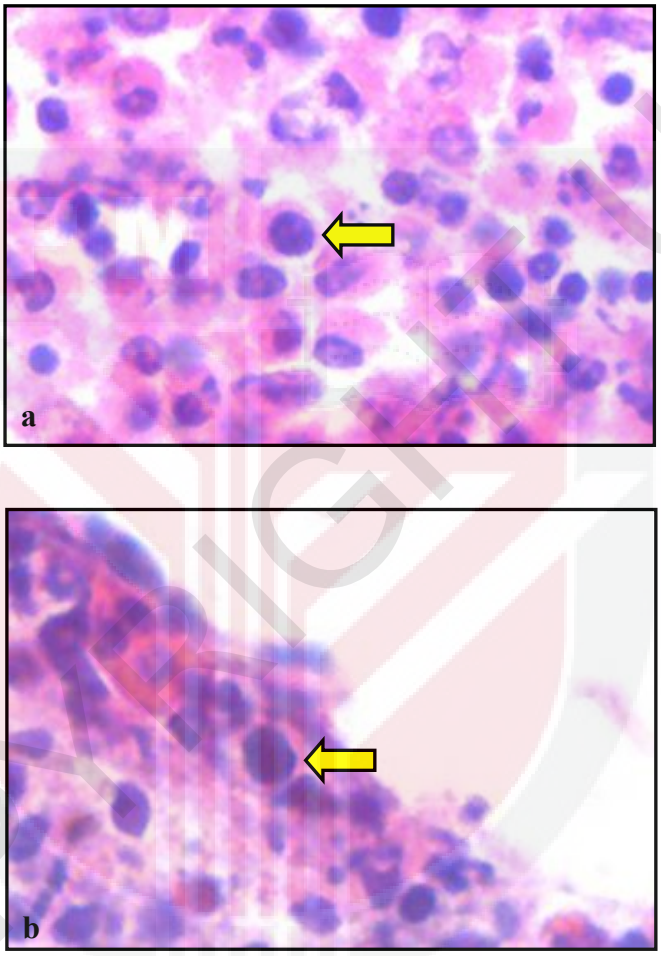

Figure 6: Group A. (a) Basophilic intranuclear inclusion body in gizzard of embryo at day 6 pi, and (b) large intranuclear inclusion body in CAM of embryo at day 6 pi. HE, 1000x.

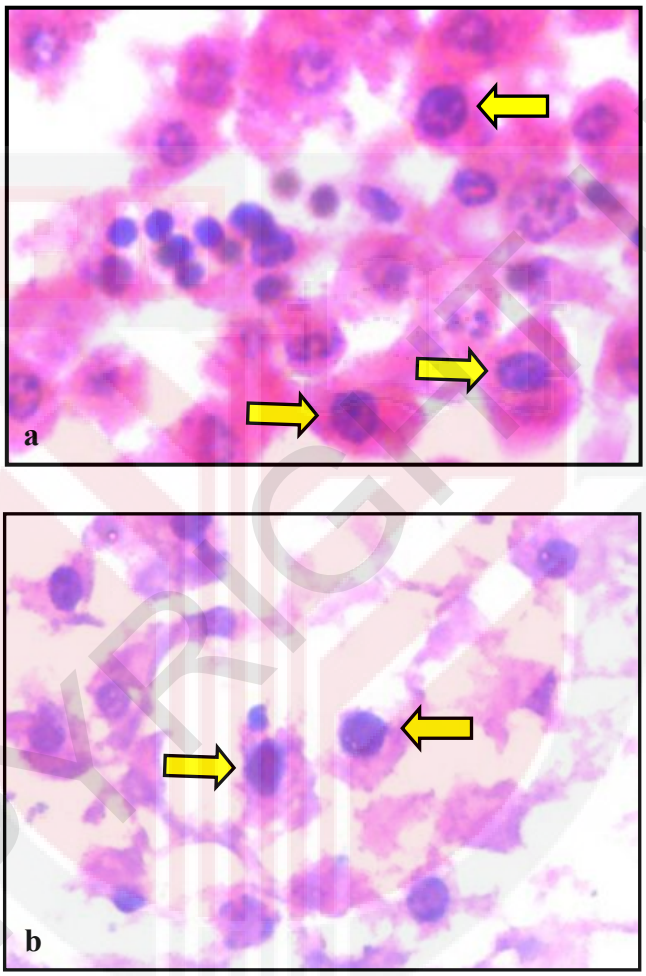

Figure 7: Group B. (a) Mild to moderate degeneration and necrosis with intranuclear inclusion bodies in hepatocytes of embryo at day 4 pi, and (b) extensive degeneration and necrosis with large basophilic intranuclear inclusion bodies in hepatocytes of embryo at day 7 pi. HE, 1000x.

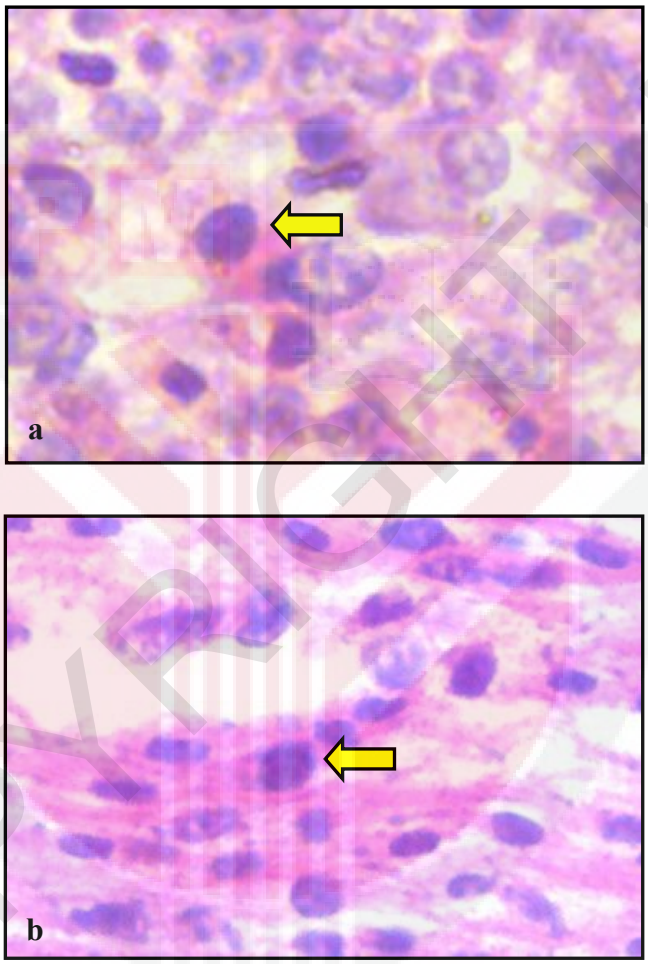

Figure 8: Group B. (a) Intranuclear inclusion body in gizzard of embryo at day 4 pi, and (b) large basophilic intranuclear inclusion body in gizzard of embryo at day 6 pi. HE, 1000x.

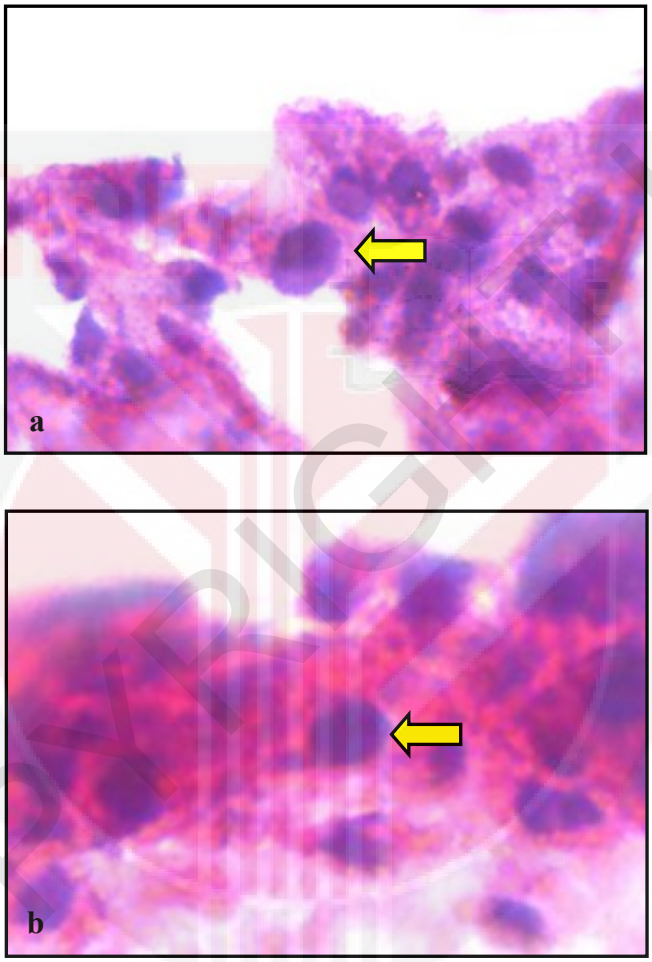

Figure 9: Group B. (a) Intranuclear inclusion body in chorionic epithelium of CAM at day 3 pi. HE, 1000x and (b) large intranuclear inclusion body in chorionic epithelium of CAM at day 7 pi. HE, 400x.

DISCUSSION

Pathogenicity of FAdV in SPF embryonated chicken eggs has been determined by mortality, gross and histopathological lesions of the embryos. The study demonstrated that both FAdV isolates were able to cause 100% mortality in embryos throughout the period. This was achieved on day 8 pi for group A and 7 day pi for group B. It is indicated that the isolates are highly pathogenic and able to adapt in SPF eggs. The gross lesions recorded include hepatic necrosis, hydropericardium and thickening of chorioallantoic membrane (CAM). Most cases of hydropericardium syndrome has been associated with FAdV-4 (Adair and Fitzgerald, 2008). In this study, hydropericardium also was recorded, but only in one embryo at day 7 pi.

The histopathological lesions include the presence of basophilic intranuclear inclusion bodies in hepatocytes, CAM and gizzard as well as degenaration and necrosis of the hepatocytes. The presence of basophilic intranuclear inclusion bodies is associated with active replication of the virus in the nucleus as the virus particles were detected only in cells with basophilic inclusions. On the other hand, eosinophilic inclusions contain only fibrillar granular material and filaments, which may represent a degenerative process or an early stage in the formation of virus (Smyth and McNulty, 2008 ; Hair-Bejo, 2005).

Not all FAdV serotypes are able to adapt and cause the recognizable lesions in the

embryonated chicken eggs (Juliana *et al.,* 2014). The majority of FAdV isolates have been isolated in eggs are FAdV-1 and FAdV-5 (McFerran, 1991). Recently, FAdV-9 has been proven to be able to adapt and pathogenic to SPF embryonated chicken eggs (Alemnesh *et al.,* 2012). In this study, both UPM 11134 and UPM 1127 FAdV isolates were successfully isolated in embryonated chicken eggs. Virus isolation for inclusion body hepatitis is commonly performed via cell culture as an alternative, either on chicken embryo liver (CEL) or kidney cells.

FAdV is believed to act as a secondary pathogen, following infection with an immunosuppressive agent, such as infectious bursal disease virus (IBDV), chicken infectious anaemia virus (CAV), birnavirus, circovirus, mycoplasma or exposure to mycotoxin (Hess, 2000; Philippe *et al.,* 2005 ; Adair and Fitzgerald, 2008). However, FAdV has been reported as the primary pathogen of IBH (Philippe *et al.,* 2005). In this study, it is highly suggestive both isolates causing IBH disease since the liver, CAM and gizzard of the embryos were severely affected based on gross and histopathological lesions.

IBH causes high mortality in chickens, thus resulting a significant impact on the poultry industry in Malaysia. Therefore, vaccine is currently needed to protect chickens and prevent vertical transmission to embryonated eggs. Commercial live vaccine containing FAdV-8b strain has been used in Australia to vaccinate broiler breeders. This vaccines had effectively controlled the vertical transmission of FAdV, subsequently

prevent the outbreaks of IBH (Steer *et al.,* 2011). Vaccinating the broiler breeders twice with an autogenous killed vaccine have been successfully demonstrated to protect broilers against an experimental challenge of IBH due to development of maternal antibody (Alvorado *et al.,* 2007). Furthermore, vaccination using inactivated oil emulsion has also been explored for the prevention of IBH outbreaks.

However, vaccination against this disease specifically for both isolates in Malaysia is currently not available. Thus, virus for both isolates can be attenuated by passaging in embryonated chicken eggs or cell culture for development of vaccine against IBH. Live poultry vaccines induce a variety of innate and adaptive immune responses, while inactivated vaccines are most commonly used to vaccinate layer and breeder chickens, to boost immunity against many diseases following priming with live vaccines, and to confer protection in progeny by transfer of maternal Ab (Schijns *et al.,* 2008). Inactivated vaccines contain high concentrations of virus, chemically inactivated by formalin or betapropiolactone, are commonly formulated with adjuvants, and are administered by subcutaneous or intramuscular injection (Glisson and Kleven, 1993).In addition, diagnostic kit such as Enzyme-linked Immunosorbent Assay (ELISA) can also be developed with coated virus isolate antigen for future diagnosis of this disease. Since the lesions are more severe in liver, it is suggested that liver sample is more suitable in isolating FAdV.

CONCLUSION

Isolate A (UPM 11134) and isolate B (UPM 1127) of the recent FAdV isolates of Malaysia are highly pathogenic in SPF embryonated chicken eggs. Cumulative mortality of 100% was recorded at days 7 and 8 pi for the isolate A and isolate B, respectively. The typical gross and histological lesions of IBH were observed include thickened CAM and enlarged, pale or congested liver with multifocal areas of necrosis. Basophilic intranuclear inclusion bodies in hepatocytes, CAM and gizzard as well as degenaration and necrosis of the hepatocytes were recorded histologically. The FAdV isolates were successfully isolated and adapted in embryonated chicken eggs via CAM route, thus provide an alternative to tissue culture for FAdV isolation.

RECOMMENDATIONS

Since the pathogenesis of this disease is still remain unclear, further study should be conducted to determine the pathogenesis of IBH in both SPF chickens and embryonated chicken eggs. As for future vaccine development, further passaging of these virus isolates can be performed either in embryonated chicken eggs or cell culture.

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APPENDIX 1

Experimental design for pathogenicity study of two FAdV isolates

Groups	Total SPF eggs
A	**12**
B	**12**
C	**15**
Total SPF eggs used in this study	**39**

Group A: UPM11134 FAdV isolate

Group B: UPM1127 FAdV isolate

Group C: Control

APPENDIX 2

Virus propagation technique in specific pathogen free embryonated chicken eggs: routes of inoculation (Senne, 1989)

Chorioallantoic membrane (CAM) inoculation

1) Candle the embryonating eggs and markthe side of the eggs approximately midway along the long axis where the vein structure is well-developed.
2) Place the egg horizontally on egg flat and disinfect both the air-cell end and the side of the egg that has been marked in step 1 above.
3) Drill a small hole through the eggshell and eggshell membrane at the centre of the air cell. Drill a second hole on the side of the egg.
4) Then the egg is taken into a dark room to candle and observe the dropping of the CAM. While holding the egg against the egg candler, apply vacuum by using a suction bulb to the hole at the air cell, withdrawing the air from the air cell will cause the CAM to drop, thus forming a new false air cell directly over the CAM.
5) Using a syringe with 25-gauge 5/8 inch (16mm) needle, inoculate 0.1 inoculum per egg by inserting the needle vertically just inside the egg shell and injecting the desired amount.
6) Seal the hole, and rock the egg gently to distribute the inoculum evenly over the CAM surface,and return the egg into incubator. The eggs were placed horizontally to prevent the false air cell from shifting.

APPENDIX 3

Cumulative mortality of SPF eggs of group A and B via CAM route

Group	Total no.of SPF eggs	Cumulative Mortality								Mortality (%)
		Day (pi)								
		1	2	3	4	5	6	7	8	
A	12	0[a]/12[b]	0/12	8/12	10/12	10/12	11/12	11/12	12/12	100
B	12	0[a]/12[b]	0/12	3/12	8/12	8/12	9/12	12/12	-	100

a: Total number of eggs dead b: Total number of eggs inoculated

~ The SPF eggs in the control group remained normal throughout the trial.