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Research Article

Metabolic Disturbances, Histopathological Analysis, and Virus Detection in Chicken Following Infection with Fowl Adenovirus Serotype 8B

Norfitriah Mohamed Sohaimi[1,2]*, Muhammad Aidil Haikal Bin Mohd Dzilan[1], Mohd Hair Bejo[2,3], Azalea Hani Othman[1]

1. Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
2. Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
3. Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

*Corresponding author: fitriahsohaimi@upm.edu.my

ABSTRACT

Fowl adenovirus (FAdV) serotype 8b is a causative agent of inclusion body hepatitis (IBH) in chickens with serious economic impact due to huge mortality in commercial farms. To date, analysis of metabolic disturbances caused by FAdV serotype 8b infection in chicken is scanty although FAdV is highly pathogenic in specific pathogen free (SPF) chicken. It is important to highlight the effect of the FAdV infection for better control strategy against the disease. The objective of the study is to determine the metabolic parameters, virus detection, and histopathological analysis of chickens following infection with FAdV serotype 8b. Fifteen-day-old SPF chicks were divided into two groups namely, the infected group and the control group. All chicks in the infected group were inoculated with 1 mL FAdV isolate, UPM1901, via oral route at day 7 of age, while all chicks in the control remained uninoculated. At 0, 3, and 7 days post-inoculation (dpi), blood and serums were collected for clinical biochemistry analysis followed by histological examination and virus detection using polymerase chain reaction (PCR). Marked anemia with an elevation of aspartate-aminotransferase (AST) and gamma-glutamyl transferase (GGT) levels in infected chickens at 3 and 7 dpi with low creatine kinase levels than control group were recorded due to liver damage caused by FAdV infection. Swollen, hemorrhage, and necrosis of the liver were observed in chicken from the infected group at 3 and 7 dpi with evidence of basophilic intranuclear inclusion bodies in degenerated hepatocytes. Liver, kidney, and lymphoid organs were positive for FAdV at 3 and 7 dpi in the infected group. It was concluded that FAdV serotype 8b isolate UPM1901 induces metabolic disturbances and histopathological lesions with detectable viral nucleic acid in the liver and lymphoid organs which necessitates effective therapeutic strategy for liver protection and a better immune response against IBH disease.

Key words: Fowl adenovirus (FAdV), histopathological, liver enzymes, metabolic, serotype 8b, virus detection

INTRODUCTION

Fowl adenoviruses (FAdVs) are non-enveloped double-stranded DNA viruses, which belong to the genus *Aviadenoviridae*, under the *Adenoviridae* family and they can be spread vertically and horizontally (Schachner *et al*., 2018). FAdVs can be classified into five different species which are FAdV-A to FAdV-E and have 12 serotypes which are FAdV-1 to -8a and 8b to -11 (Sabaruddin *et al*., 2021). FAdVs are common infectious agents in fowl worldwide. Typical FAdV infections in chickens are inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and adenoviral gizzard erosion (AGE) caused by pathogenic FAdV strains, and it led to economic losses on a global scale (El-Shall *et al*., 2022).

Virulence FAdV caused high mortality and severe lesions in multiple organs with immunosuppressive effects in infected SPF chickens (Niu *et al*., 2017). In Malaysia, the IBH outbreak was first reported in Perak state in 2005 and subsequently in other states as reported by previous works (Norina *et al*., 2016; Sohaimi *et al*., 2022). FAdV serotype 8b was identified as the primary pathogen of IBH and highly pathogenic in SPF chickens (Sohaimi *et al*., 2019). Gross and histopathological lesions of affected chickens indicated that liver lesions were predominant, particularly in IBH cases. The liver exhibited enlargement, pale, and friable, with histopathological findings revealing multifocal inflammation and necrosis (Tsiouris *et al*., 2022). Additionally, characteristic large basophilic intranuclear inclusion bodies were observed in hepatocytes (Tsiouris *et al*., 2022). The liver is the major tropism for FAdV replication and caused severe damage as it was characterized by a high level of coxsackievirus-adenovirus receptor (CAR) expression and integrin receptors that bind to adenovirus fibers, serve as a trigger for infection (Wang *et al*., 2023). To date, a molecular technique using polymerase chain reaction (PCR) and sequencing based on the hexon gene is useful for virus detection since this protein being the major gene of the adenovirus, is recognized for harboring the neutralizing epitope (Wang *et al*., 2017; Islam *et al*., 2023).

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Recently, the poultry industry has faced significant economic losses primarily due to high mortality rates and reduced production on commercial farms, all attributed to IBH triggered by FAdV serotype 8b. The liver is the major tropism for FAdV replication as reported worldwide and resulted in severe lesions caused by all 12 serotypes with sudden death in the infected host. However, there is still a lack of information on the impact of FAdV serotype 8b on metabolic disturbances and pathological changes in various organs in chickens which contribute to the occurrence of disease outbreaks and mortality. This knowledge is crucial to emphasize the disease process involving blood and serum biochemistry profiles following natural infection in chickens. In addition, understanding on parameters for liver damage caused by FAdV will provide a clear indication of disease impact and subsequently, is important for effective treatment strategy against the outbreak in a local poultry farm. The objective of this study is to evaluate the metabolic parameters and histopathological analysis and in addition to detect the presence of FAdV in liver and lymphoid organs by molecular method in chickens following inoculation with FAdV serotype 8b isolate UPM1901.

MATERIALS AND METHODS

Virus isolate

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

Experimental design and sampling protocol

The animal study was conducted under the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia with approval number: UPM/IACUC/AUP-U015/2023. Fifteen (15) day-old chicks were divided into 2 major groups namely, FAdV infected group and the control group. Six (6) chicks were assigned to FAdV infected group and nine (9) chicks to the control group. All chicks in the infected group were inoculated with 1 mL FAdV isolate, UPM1901, at virus titer $10^{7.1}TCID_{50}$/mL via oral route at 7 days old. All chicks in the control group remained uninoculated and were used as a control group in this study. All chickens were monitored throughout the trial and both feed and water were given *ad libitum* until 7 days post-inoculation (dpi). At 0 dpi, body weight was recorded prior to necropsy, and whole blood, and serum were collected for complete blood count (CBC) and serum biochemistry analysis from three chicks in the control group for health status monitoring prior to sacrifice by cervical dislocation. Samples of liver, thymus, caecal tonsil, kidney, bursa of Fabricius, spleen, and bone marrow were collected from the chicks for virus detection via PCR analysis and histological examination (Sohaimi *et al*., 2021). In addition, livers were weighed for all chickens following necropsy. Sampling was performed subsequently in both groups at 3 dpi and 7 dpi.

Serum biochemistry

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

Gross and histological examinations

Upon necropsy, any gross lesion was recorded in both infected and control groups. A sample of the liver was harvested and fixed in 10% buffer formalin for histological examination (Matos *et al*., 2016). All samples were analyzed and described based on the severity of the lesions (Sohaimi *et al*., 2021).

Molecular detection by conventional PCR

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

Statistical analysis

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

RESULTS

Clinical signs

FAdV-infected chicks showed clinical signs associated with IBH such as lethargy, depression, ruffled feathers, inappetence, and watery greenish dropping starting from 2 dpi onwards without mortality throughout the trial. Whereas there is no clinical sign was observed for control chickens.

Mean body weight

At 0 dpi, the mean body weight for the control group was 60.3 g ± 1.2 followed by 58 g ± 6.7 and 64.3 g ± 1.8 at 3 dpi and 7 dpi, respectively. The mean body weight for FAdV infected group was 83.6 g ± 4.6 which increased high (p<0.05) at 3 dpi compared to the control group (Table 1). At 7 dpi, the FAdV infected group was 73.7 g ± 11.4 without significant difference (p>0.05) compared to the control group.

Table 1. Mean of body weight of chickens in control groups and FAdV-infected groups throughout the study

	Mean ±SEM[a] of body weight		
Group/Sampling Day (dpi)[b]	0[c]	3	7
Control	60.3g ± 1.20	58g ± 6.7	64.3g ± 1.8
FAdV infected group	NA[d]	83.6g ± 4.6*	73.7g ± 11.4

*p<0.05

[a]SEM: Standard Error Mean

[b]dpi: day post-inoculation

[c] : Sampling at 0-day post-inoculation (dpi) was performed only in the control group to monitor the health status of the chickens prior to inoculation.

[d]NA: Not available.

Red Blood Cells (RBC) parameter

At 0 dpi, RBC concentration and PCV in the control group was 1.5 ± 0.4 and 25%, respectively. High RBC concentration at 3 dpi was recorded in an infected group compared to the control group. In contrast, the concentration at 7 dpi was significantly lower (p<0.05) in the infected group which was 1.6 ± 0.1 than the normal range at 2.5 – 3.5 x 10^6 µL (Figure 1). Similarly, for packed cell volume (PCV), FAdV infected group produced 18% concentration which was significantly lower (p<0.05) than the normal range at 22 – 35% as recorded in the control group at 28% at 7 dpi (Figure 2). However, there was no significant difference (p>0.05) at 3 dpi. There were also no significant differences (p>0.05) in mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) between the control group and the infected group.

Serum biochemistry analysis for AST, GGT, and CK

At 0 dpi, the level of AST, GGT, and CK were recorded at values 435.6 ± 149, 14 ± 1.5, and 282.5 ± 156, respectively, in the control group. AST levels increased in the infected group which were 851.7 ± 436 and 795.4 ± 494 at 3 dpi and 7 pi, respectively than the control group at levels 525.2 ± 1 and 32.3 ± 88, (Figure 3). However, CK level was lowered in the infected group which was 1108.2 ± 1095 at 3 dpi than the control group. In addition, the CK level of the infected group was 791.5 ± 785 which was also lower as compared to the control group which was 1174.6 ± 139 at 7 dpi (Figure 4). Lastly, the GGT level was increased in the infected FAdV group which were 15 ± 6.5 and 24 ± 4.5 at 3 dpi and 7 dpi, respectively as compared to the control group which was 22.5 ± 11 at 7 dpi.

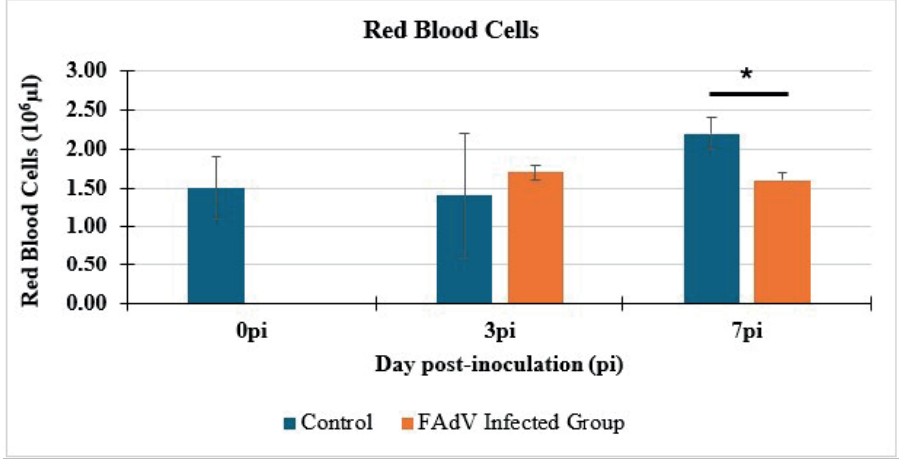

Fig. 1. Concentration of red blood cells (RBC) between control and FAdV infected groups throughout the trial. RBC concentration in the FAdV-infected group at 7 dpi was low significantly (p<0.05) compared to the control group. *asterisk indicates significant differences between groups.

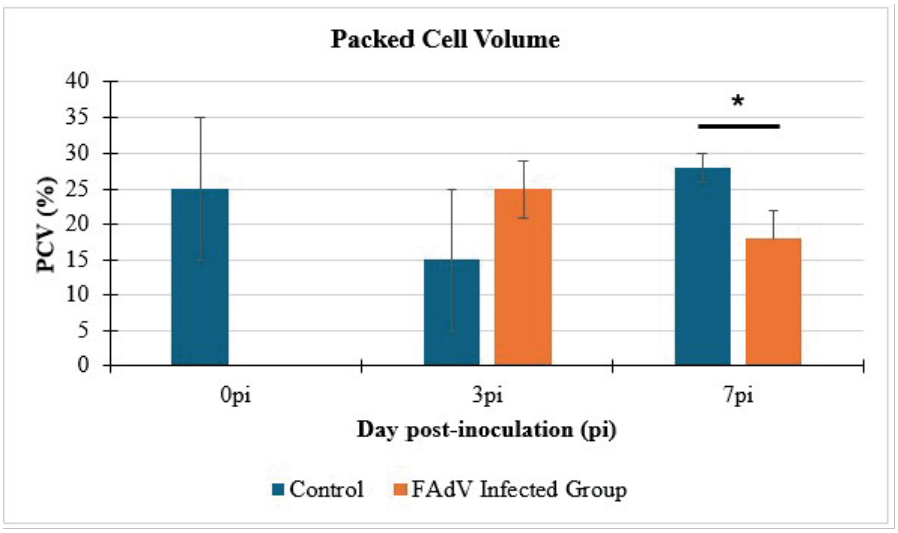

Fig. 2. Mean concentration of packed cell volume (PCV) between control and FAdV infected groups throughout the trial. PCV concentration in the FAdV-infected group at 7 dpi was low significantly (p<0.05) compared to the control group. *asterisk indicates significant differences between groups.

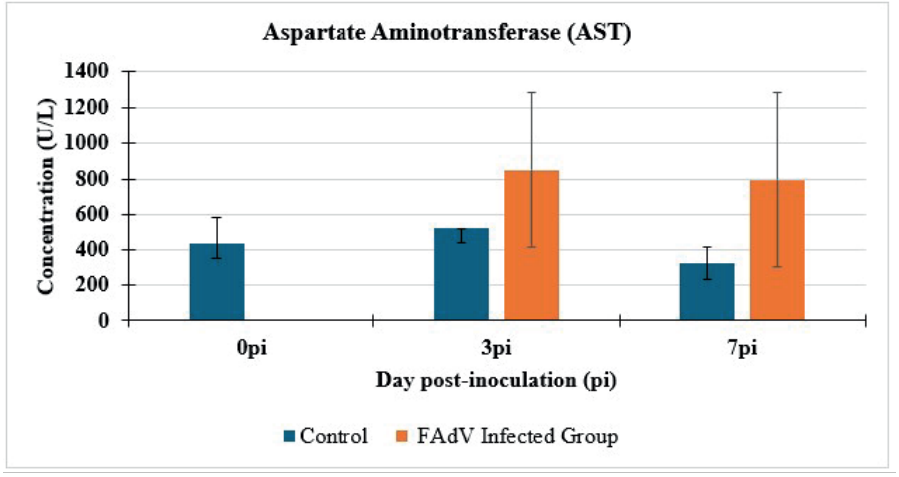

Fig. 3. Mean concentration level of aspartate aminotransferase (AST) enzyme between control and FAdV-infected groups of chickens throughout the trial.

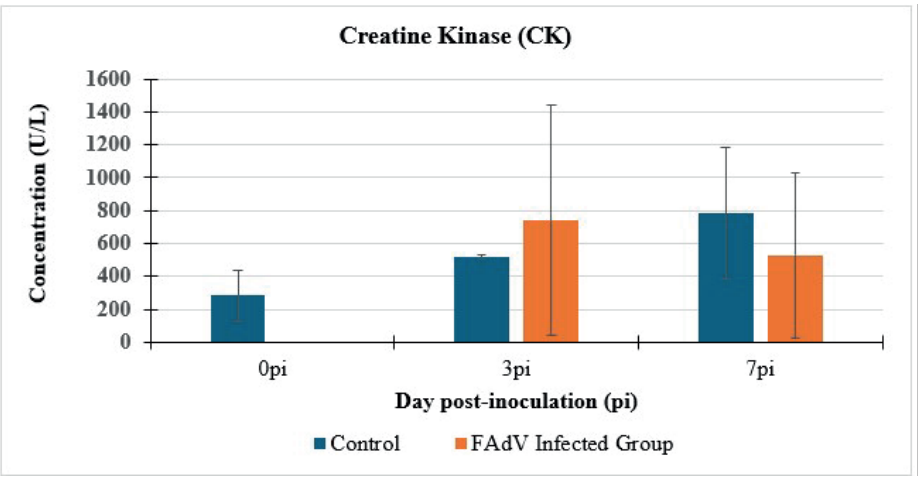

Fig. 4. Mean concentration level of creatine kinase (CK)enzymes between control and FAdV-infected groups of chickens throughout the trial.

Gross lesions

All livers were normal in the control chickens at 0, 3, and 7 dpi with brownish coloration and glistening appearance. In the infected groups, the liver was swollen, enlarged, yellowish discoloration, congested, and friable at 3 dpi without significant changes in other tissues. Similarly, marked gross changes were observed at 7 dpi in the liver with petechial hemorrhages, yellowish discoloration, sharp edges, friable, and necrosis (Figure 5) along with pale, swollen, and urate deposition in the kidney.

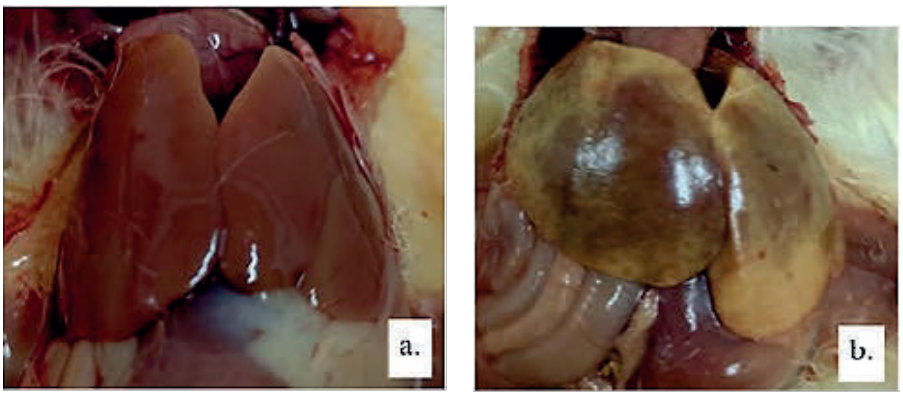

Fig. 5. Necropsy finding of the liver in chickens between control and FAdV infected groups at 7 days post-inoculation. **(a):** Normal liver in the control group with brownish coloration and glistening appearance, **(b):** Liver of FAdV infected chicken with swollen, pale with yellowish discoloration, petechial hemorrhages, sharp edges, friable and necrosis after inoculated with FAdV-8b isolate UPM1901.

Liver weight

The mean liver weight for control chickens was 3.7 g ± 0.7 at 0 dpi, followed by 2.8 g ± 0.2 at 3 dpi and 2.4 g ± 0.1 at 7 dpi. The mean liver weight of chicken for the infected group was high significantly (p<0.05) at 3 dpi which was 5.3 g ± 0.1 and 7 dpi which was 5.6 g ± 0.6 as compared to the control group (Figure 6).

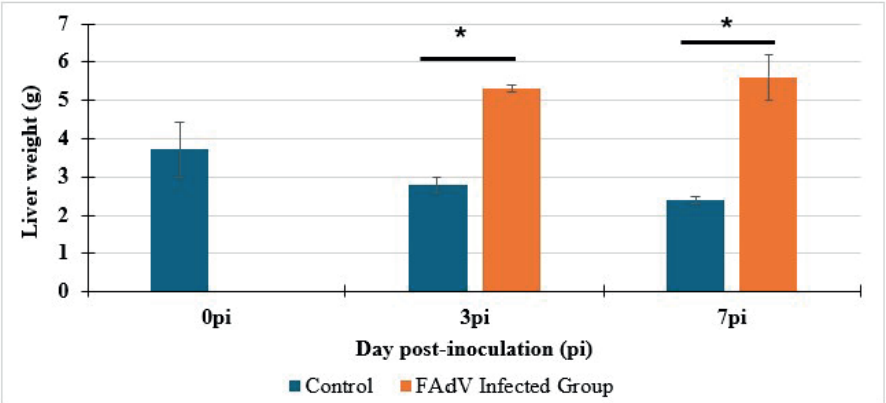

Fig. 6. Mean liver weight of chickens between control and FAdV infected groups following inoculation with UPM1901 isolate at 7-day-olds. Liver weight in FAdV infected group at 3 dpi and 7 dpi were high significantly (p<0.05) compared to the control group. *asterisk indicates significant differences between groups.

Liver weight to body weight ratio

Liver body weight ratio at 0 dpi was 0.06 g ± 0.01 in the control group. The ratio for the FAdV infected group was significantly higher (p<0.05) at 3 dpi and 7 dpi which were 0.07 g ± 0.003 and 0.08 g± 0.01 respectively as compared to the control group with ratios 0.05 g ± 0.003 and 0.04 g ± 0.003, respectively (Table 2).

Table 1. Mean of liver weight to body weight ratio of chickens in control groups and FAdV infected groups throughout the study

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

*p<0.05

[a]SEM: Standard Error Mean

[b]dpi: day post-inoculation

[c] : Sampling at 0-day post-inoculation (dpi) was performed only in the control group to monitor the health status of the chickens prior to inoculation.

[d]NA: Not available.

Histopathological findings

Few basophilic intranuclear inclusion body (INIB) in the hepatocytes was observed at 3 dpi and 7 dpi of the infected group (Figure 7). In contrast, there is no significant finding in the control group for all tissues at 0, 3, and 7 dpi.

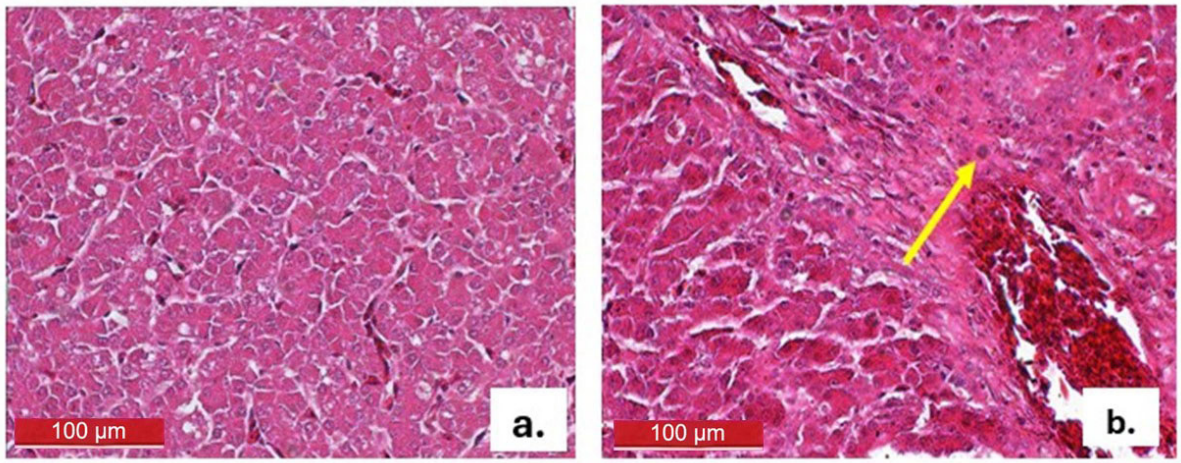

Fig. 7. Histopathological findings of the liver between control and FAdV infected groups following inoculation with UPM1901 isolate at 7-day-olds. **(a).** Normal liver in the control group, **(b).** presence of basophilic intranuclear inclusion bodies (INIB) as indicated as a yellow arrow for the infected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

Virus detection by conventional polymerase chain reaction (PCR)

All the tested organs such as liver, kidney, and lymphoid tissues including thymus, spleen, bursa of Fabricius, bone marrow, and caecal tonsils were positive to FAdV at 3 dpi and 7 dpi with expected PCR size product 897 base pairs (bp) (Figure 8).

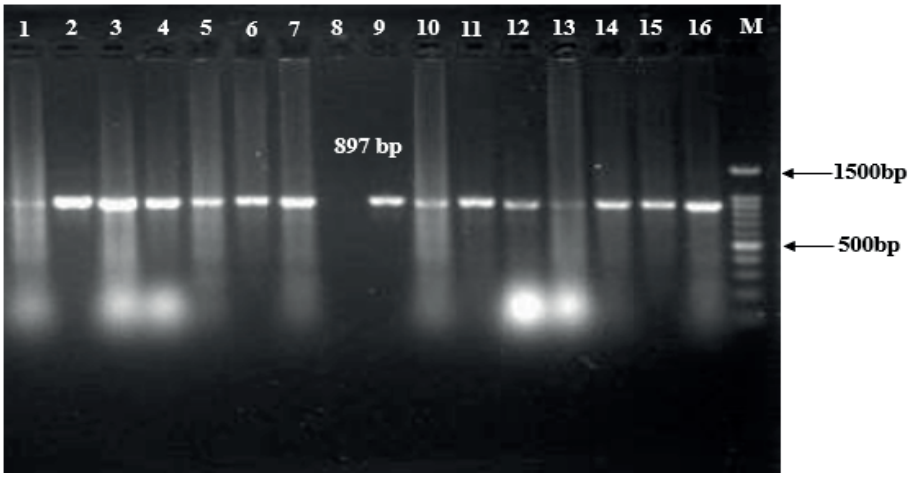

Fig. 8. Electrophoresis of PCR product in 1.5% agarose gel amplifying nucleic acid of hexon gene of fowl adenovirus with expected PCR size product at 897 base pairs (bp). Lane M: 100 bp DNA marker, Lane 8: Negative control, Lane 9: Positive control (UPM1137), Lane 1-7: Organs from day 3 post-inoculation (pi) positive to FAdV, Lane 1: Thymus, Lane 2: Spleen, Lane 3: Liver, Lane 4: Kidney, Lane 5: Bursa of Fabricius, Lane 6: Bone Marrow, Lane 7: Cecal Tonsil, Lane 10-16: Organs from day 7pi positive to FAdV, Lane 10: Thymus, Lane 11: Spleen, Lane 12: Liver, Lane 13: Kidney, Lane 14: Bursa of Fabricius, Lane 15: Bone marrow, Lane 16: Cecal tonsil.

DISCUSSION

It was demonstrated that FAdV serotype 8b isolate UPM1901 given by oral route at day 7 old induces clinical signs shown of lethargy, depression, ruffled feathers, inappetence and watery greenish diarrhea in SPF chickens started from 2 dpi onwards without any mortality throughout the trial. The results were consistent with a previous study by Cizmecigil *et al*. (2020). In contrast, all chickens in the control group were normal throughout the trial, however, the mean body weight was lowered than the infected group probably due to reduced feed consumption which could triggered by low lighting intensity. While mean body weight for the infected group was increased significantly ($p<0.05$) starting at 3 dpi compared to the control group. This finding could be due to age-related resistance against FAdV as indicated by Cook *et al*. (1974) and Matos *et al*. (2016) since all chickens were inoculated at day 7 old. As compared to the previous work, high mortality was recorded in SPF chickens after inoculation at 1 day old (Mohamed Sohaimi *et al*., 2019). It is believed that evidence of age-related resistance is observed with avian adenoviruses as the host's age rises, there is a notable limitation in the viral multiplication within the host, leading to a decrease in mortality (Rahimi & Haghighi, 2015).

The analysis of CBC parameters at 3 dpi indicated that the concentration of RBC and the PCV were higher in the infected group compared to the control group. This discrepancy is likely attributable to dehydration in the chickens, which may resulted from

reduced water intake in the affected birds. As the disease progressed until 7 dpi, significantly lower (p<0.05) RBC concentrations and PCV values were recorded in an infected group than in the control chickens. There is marked anemia with 18% PCV value in chickens infected with FAdV serotype 8b due to a reduction of RBC synthesis with affected bone marrow. These findings were consistent with previous works in which FAdV infection induced dehydration, exhaustion with pale comb, and shank in infected chickens (Alzuheir *et al*., 2022; Levkutova *et al*., 2023).

Serum biochemistry analysis showed an elevation of AST level in the infected chickens at 3 dpi and 7 dpi with low CK levels indicating that liver damage due to FAdV infection. It was consistent with a higher GGT level at 3 dpi and 7 dpi than the control group which indicates that this finding is highly related to liver and another biliary compromise such as hepatobiliary diseases as reported by previous studies (Metra *et al*., 2022; Sang *et al*., 2023). Current findings were compatible with previous work reported by De Luca *et al*. (2020). Although AST activity is considered a very sensitive enzyme, it is not a specific indicator for liver injury since a high level of AST could be interpreted due to muscle damage as well. Thus, AST level must be compatible with CK level to differentiate between liver and muscle damage.

Both gross and histopathological lesions were predominantly found in the liver as this organ was the major tropism for FAdV replication and caused severe damage. At 3 dpi and 7 dpi, the liver of the infected group was swollen, enlarged, congested, and friable with evidence of few basophilic INIB in the hepatocytes under microscopically. It showed that the mean liver weight in the infected group is significantly higher (p<0.05) than the control group at 3 dpi and 7 dpi which is consistent with gross findings due to hepatitis and swollen livers. Analysis of the liver weight-to-body weight ratio is crucial in this trial for determining liver enlargement proportionate to body weight. As a result, a significant (p<0.05) higher liver weight-to-body weight ratio at 3 dpi and 7 dpi was recorded in an infected group than control group. These findings are compatible with previous studies conducted by Sohaimi *et al*. (2019) and Norina *et al*. (2016). Moreover, there were no abnormal findings in other tissues at 3 dpi during acute infection, however, at a later stage, swollen, pale, and urate deposition in the kidney were noticed in the infected chickens at 7 dpi which was similarly found with other previous work (Tsiouris *et al*., 2022).

Conventional PCR was applied for this study as a molecular detection by amplifying the hexon gene. Hexon protein is the most important part of the adenovirus proteome to enable the classification and recognition of individual serotypes (Ebner *et al*., 2005). This protein also plays a major role in virus infectivity and tissue tropism (Sohaimi & Bejo, 2021). It was demonstrated that all lymphoid organs, liver, and kidney were positive for FAdV at 3 dpi onwards. This finding indicates that the virus multiplied in various tissues within the incubation period following inoculation via the oral route. According to previous literature, IBH can be classified into three stages based on the presence and severity of hepatic lesions: incubation (1–3 dpi), degeneration (4–7 dpi), and convalescence (14 dpi) (Steer *et al*., 2015). Thus, in the present finding, the virus was present in the targeted organs from 3 dpi. This phase corresponds to the incubation period, in which fast viremia and virus multiplication were succeeded in the targeted organs (Steer *et al*., 2015; Matos *et al*., 2016).

In this study, it was demonstrated that FAdV serotype 8b caused anemia and metabolic disturbances based on elevated liver enzymes following oral inoculation at 7-day-old chicken. In addition, the isolate induced significant gross and histopathological lesions resulting in severe damage to the liver and lymphoid organs. These findings discovered the impacts of FAdV serotype 8b in chickens, highlighting the disease mechanism that primarily targets systemic and liver tissues. This targeting ultimately resulted in liver failure and acute mortality, as documented in earlier research (Sabarudin *et al*., 2021). It`s indicated that the disease could be prevented by various approaches such as liver therapy and immune boosters to the commercial poultry flock.

CONCLUSION

FAdV serotype 8b isolates UPM1901 induced metabolic disturbances and histopathological lesions in chickens with detectable viral nucleic acid in liver and lymphoid organs. Thus, it urgently needs an effective therapeutic strategy for liver protection and better immune response against IBH disease in the poultry industry.

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ETHICAL STATEMENT

This study was approved by under Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia with approval number: UPM/IACUC/AUP-U015/2023.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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