P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com
Research Article https://doi.org/10.47278/journal.ijvs/2022.219

Pathogenicity of Avian Adenoviruses Type D and Type E Isolated from Field Cases of Inclusion Body Hepatitis–Hydropericardium syndrome (IBH–HPS) in Broiler chickens in Egypt

Gomaa A. Abdel-Alim[1], Mostafa G. Naguib[1],*, Diaa G. Khelfa[1], Marwa S. Khattab[2] and Eman Anter Morsy[1]

[1]Department of Poultry diseases, Faculty of Veterinary Medicine, Cairo University, Cairo 12211, Egypt
[2]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Cairo 12211, Egypt
***Corresponding author:** mostafa.vet355@cu.edu.eg

Article History: 22-716 Received: 01-Oct-22 Revised: 20-Nov-22 Accepted: 27-Nov-22

ABSTRACT

In this study, 210 one-day-old commercial broiler chicks were used to investigate the pathogenicity of two types of Avian Adenoviruses (type D and type E) recently isolated from field cases. These chicks were divided into four groups; birds of group 1 (G1) and group 2 (G2) were experimentally inoculated with two local isolates of fowl aviadenovirus FAdV D-2 and FAdV D-11, respectively, while chicks of group 3 (G3) were experimentally inoculated with FAdV-E 8a. All birds of these three groups were experimentally inoculated orally at one-day of age with a dose of 0.5mL (10^7 TCID50/mL). Birds of group 4 (G4) served as uninoculated blank controls. Clinical signs, gross lesions and histopathological changes were examined at different intervals post inoculation. Birds of all infected groups showed hydropericardium, focal hepatic necrosis and ecchymotic hemorrhage of thigh and breast muscles. Hepatitis, glomerulonephritis, pericarditis, myocarditis and lymphoid depletion in the spleen were the main histopathological observations. Mortality rates of 12, 16 and 6% were observed in chicks of G1, G2 and G3, respectively, indicating that FAdV-D isolates were more virulent than FAdV-E. Hemagglutination inhibition test revealed significant decrease in antibodies titers against inactivated NDV vaccine in birds of the three FAdVs infected groups compared to the chicks of the control group. In Egypt, several studies reported Adenoviruses infections in broilers; however, to our knowledge this is the first study in which the pathogenicity of FAdVs isolates has been reported. It was concluded that FAdV-D 2, FAdV-D 11 and FAdV-E 8a are primary agents, in addition to possibility of these isolates to act as neglectable immunosuppressive agents incriminating to vaccination failure in chickens.

Key words: FAdVs, Broiler Chickens, Inclusion Body Hepatitis, Hydropericardium, Pathogenicity, Histopathology, PCR.

INTRODUCTION

Different fowl aviadenoviruses (FAdVs) serotypes have been isolated from inclusion body hepatitis/ hydropericardium syndrome (IBH/HP) outbreaks. Fowl aviadenoviruses are naked viruses placed under genus Aviadenovirus within the family Adenoviridae. Depending on their serological relationship, 12 FAdVs serotypes have been identified and divided into five groups FAdVs A-E, according to their genomic length (Hafez 2011). Broilers and layers have both been observed to have IBH/HP outbreaks (Hafez 2011). Chickens 3-6 weeks of age are more susceptible to IBH/HP infection, with high mortalities up to 30% (Schachner et al. 2021). This infection can spread vertically, as well as horizontally. The ability of fowl adenovirus (FAV) to be transmitted from parent birds to offspring is indeed a key feature (McFerran et al. 2000; McFerran et al. 1977). Infected breeders can transmit the virus to their offspring for 3-6 weeks until they gain immunity (Del Valle et al. 2020; Toro et al. 2001). The virus is transmitted horizontally in a flock by the oral-faecal route, with further dissemination occurring through mechanical mechanisms and contamination with infected faeces (Cook 1983; McFerran et al. 2000; Sohaimi et al. 2021). Following experimentally oral inoculation of

Cite This Article as: Abdel-Alim GA, Naguib MG, Khelfa DG, Khattab MS and Morsy EA, xxxx. Pathogenicity of Avian Adenoviruses Type D and Type E Isolated From Field Cases of Inclusion Body Hepatitis–Hydropericardium syndrome (IBH–HPS) in Broiler chickens in Egypt. International Journal of Veterinary Science x(x): xxxx. https://doi.org/10.47278/journal.ijvs/2022.219

chickens, the virus enters the intestinal epithelium within 12 hours after infection and can be found in the blood as soon as 24 hours after infection (Saifuddin et al. 1991). The virus is present in its target organs, the liver and the spleen, within 2 and 3 days after infection. This phase relates to the period of incubation, during which no clinical indications or gross lesions are noted (Steer et al. 2015). The incubation period is followed by a rapid multiplication of the virus and a viremia, which results in pathological lesions in the target organs and coincides with clinical signs of the disease (Saifuddin et al. 1991).

The birds usually recover from the disease in 7 to 9 days after infection, as evidenced by a decrease in the severity of clinical signs, as well as cellular regeneration and a reduction in viral load in the target organs (Matos et al. 2016; Steer et al. 2015). On the other hand, the virus remains dormant in the cecal tonsils and sheds in feces for a long time. The virus may be recovered from the intestine of SPF hens 12 weeks after oral infection with an FAdV-1 strain at day-old age (Gupta 2018; Jones et al. 1984).

According to our knowledge, no experimental studies have been conducted in Egypt to compare the pathogenicity of different isolated fowl adenoviruses in broiler chicken, although frequent IBH outbreaks were reported previously (Radwan et al. 2019). Recently, we isolated two different serotypes of Adenovirus type D (FAdV D-2 and FAdV D-11)) and one serotype of Adenovirus type E (FAdV-E 8a). These isolates were identified, genetically typed and registered in Gene bank (Al Naguib et al. 2021). Consequently, the present study was planned to investigate the pathogenicity of these local isolates and to confirm their role as primary pathogens in the induction of clinic-pathological lesions of IBH/HP in broiler chickens.

MATERIALS AND METHODS

Birds

A total of 210 one-day-old commercial broiler chicks (Cobb) were used in the present study. These birds were housed in separate sterilized units and were provided with feed and water *ad libtum*. The experimental procedures and handling of birds were performed following the applicable legislation of the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Cairo University, Egypt (VetCU24112020261).

Viruses

Two strains of FAdVs type D (MT386509.1, MT893206.1) and one strain of FAdVs type E (MW847902) were selected for use in this study. These viral strains were previously isolated from clinical outbreaks of IBH/HP in broiler flocks in Egypt. All isolates were identified, genetically typed and registered in gene bank (Al Naguib et al. 2021).

Virus Titration and Inoculum Preparation

The experimental strains were titrated in chicken embryo liver cells and the titer was calculated using the endpoint titration method (Muench 1937). A titer of 10^7 median tissue culture infective dose (TCID50) per mL was used to infect the experimental birds. The absence of co-infection with chicken anemia virus and infectious bursal disease virus were confirmed by using polymerase chain reaction (PCR) and the reverse transcription-PCR technique, respectively.

Experimental Design

On the arrival of experimental chicks, 10 chicks were randomly selected and euthanized. Liver, spleen, and cloacal swabs of these chicks were collected for Fowl Adenovirus detection by PCR. In addition, blood samples were collected for detection of maternal antibody against Adeno virus by Agar Gel Precipitation Test (AGPT).

The remaining 200 chicks were randomly divided into four equal groups; G1, G2, G3 and G4, with 50 chicks per group. Chicks of groups G1, G2 and G3 were orally inoculated at day-old age with 0.5 mL of 10^7 TCID50/mL FAdVs type D (MT386509.1, MT893206.1) and FAdVs type E (MW847902), respectively, while G4 served as a blank control group. The birds were vaccinated against certain destructive diseases, as given below: Inactivated ND+AIV was given at day 9 via S/C injection, NDV HB1 at day 10 via eye drop, IBDv D78 at day 12 via eye drop, and ND Lasota was given at day 18 via eye drop.

Birds of all groups were daily observed for recording clinical manifestations and mortalities. The clinical signs were scored on the basis of clinical manifestations that were recorded as: 0—Active healthy bird with no clinical signs, 1— slight depression, 2—Moderate depression with dropped wings, 3—Severe depression with ruffled feathers, 4—Reluctant to move, breathing intensely with closed eyes (Matos et al. 2016).

Three chicks from each group were euthanized at 1, 2, 3, 4, 5, 6, 7, 14-, 21-, 28- and 35-days post inoculation (DPI), samples of liver, spleen and cloacal swabs were collected and stored at –20°C for virus detection by PCR. After collection, samples of cloacal swabs were dissolved in 1mL normal saline (pH 7.4), containing streptomycin (1mg/mL), gentamycin (50g/mL), and penicillin (100,000 IE/mL), then stored until used. Similarly, samples from liver, heart, spleen and kidneys were also collected at 3, 5, 7, 14, 21, 28 and 35 DPI and preserved in 10% neutral buffered formalin for histopathological examination.

Necropsy and Histopathology

All euthanized and dead birds were examined by necropsy and gross lesions in liver, spleen, heart, intestine, muscles, kidneys and bursa of Fabricius were recorded. The collected samples of liver, heart, kidneys and spleen were fixed in 10% neutral buffered formalin, dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin blocks. Tissues of 3-4µm thickness were sectioned using a rotatory microtome (Leica 2135, Germany) and stained by hematoxylin and eosin stain, following the standard procedure. Tissue sections were examined by using a light microscope and photographed by a digital camera (Olympus XC30, Tokyo, Japan).

Nucleic Acid Extraction and FAdVs Detection by PCR

Total viral nucleic acid was extracted from 300µL of the supernatant using Genomic DNA Isolation kit (Genedirex, Catalogue No. SN026-0100), following the manufacturer's instructions. For FAdVs detection, hexon gene-specific primers (HexL1-s and HexL1-as) were used, as described previously by Raue (2005).

The targeted hexon gene was amplified in a total reaction volume of 25.0µL; 12.5µL (50% of this volume) was 2X One PCR™ polymerase chain reaction (PCR) master mix (One PCR™, Genedirex; Cat. No. MB203-0100), and 5µL 20% of the total reaction volume was DNA template, in addition to1µL of 10µM forward (HexL1-s), the same of reverse (HexL1-as) primers, and 5.5µL nuclease-free water. PCR cycling profile was achieved by one initial denaturation cycle of 4min at 94˚C, followed by 35 amplification cycles of 45s for denaturation at 94˚C, 45s for annealing at 51˚C and 1min for extension at 72˚C and a final extension stage at 72˚C for 10min. The amplified PCR products were electrophoresed and visualized under ultraviolet transillumination using 1.5% agarose gel stained with ethidium bromide.

Serology

Agar gel precipitation test (AGPT)

Agar gel precipitation test (AGPT) was performed to confirm the absence of FAdVs maternal antibodies which may interfere with the induction of infection in the experimental broiler chicks. Ten serum samples from one-day-old chicks were randomly collected and tested by AGPT using 1% Noble agar as described by Woernle (1966).

Hemagglutination Inhibition (HI) Assay

To estimate the influence of FAdVs on chicken humoral immune response, antibodies titers against NDV in NDV vaccinated chicks were measured by using the hemagglutination inhibition assay, as described previously (Chaudhry et al. 2012).

Statistical Analysis

The data has been expressed as a mean±SEM. The normal distribution of the data was verified by using the Shapiro-Wilk test, while the homoscedasticity was tested using Levene's test. One-way analysis of variance (ANOVA) test was used to determine significance of variation among groups. For multiple comparisons between groups, the Tukey's posthoc test was used. RStudiov1.3.1093 was used to create all graphs and perform statistical analyses.

RESULTS

Clinical Signs and Mortalities

During the study, birds of all groups were closely monitored for clinical signs. The results for scoring of clinical signs in birds of different groups are summarized in Table 1 and Fig. 1. The inoculated viruses induced highest mortalities in birds of G2 (16%), while G3 showed the lowest death rate (6%). No mortality was recorded in control blank group (G4) at any stage throughout the experiment. Mortality pattern in all experimental groups is summarized in Table 2 and Fig. 2.

Gross Lesions

The most prominent findings in both dead and euthanized birds were hydropericardium, pale liver with necrotic foci and subcapsular hemorrhages, enlarged and congested spleen, and petechial hemorrhages of breast and thigh muscles. Hepatitis was observed as early as 2 DPI and the severity of gross lesions increased gradually from 3 DPI to 7 DPI. However, hydropericardium affections of the liver and spleen continued throughout the experiment. Hydropericardium was severe in birds of G1 and G2 compared to G3, while hepatic damage was more prominent in birds of G3 than G1 and G2. No gross lesions were observed in any organ in birds of the control group (G4), as shown in Figs. 3 and 4.

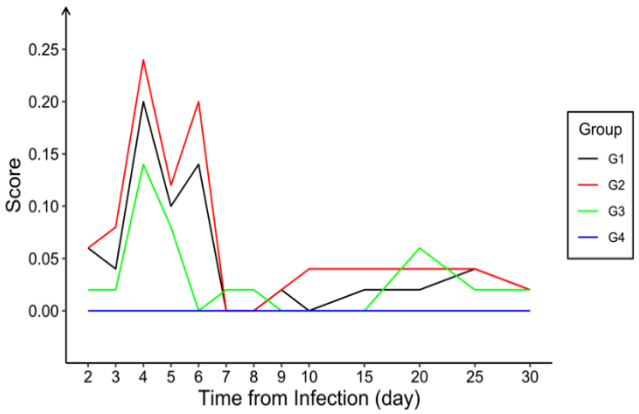

Fig. 1: Clinical signs in broiler chicks orally inoculated with FAdVs. G1, G2 and G3 were orally infected with FAdVs type D (MT386509.1, MT893206.1) and FAdVs type E (MW847902), respectively. G4 was left as a blank control group.

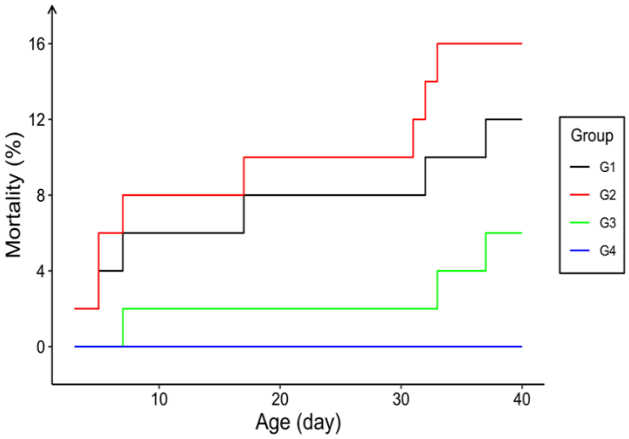

Fig. 2: Cumulative mortality percentage during the period of experiment in all groups. G1, G2 and G3 were orally infected with FAdVs type D (MT386509.1, MT893206.1) and FAdVs type E (MW847902), respectively. G4 was left as a blank control group.

Histopathology

Microscopic examination of the liver in the control group at all time intervals revealed normal histological structure. At 3 DPI, the liver revealed severe vacuolation of hepatocytes, with small eosinophilic intranuclear inclusion bodies and few periportal leukocytes infiltration in birds of G1 (Fig. 5a). In group G2, the liver revealed severe vacuolation of hepatocytes with eosinophilic intranuclear inclusions surrounded by a halo and moderate periportal mononuclear cells infiltration. Periportal hepatocytes showed karyopyknosis (Fig. 5b). In chicks of G3, the liver showed mild multifocal mononuclear inflammatory cells and severe vacuolar degeneration of

Table 1: Clinical signs scoring at different DPI in control broiler chicks and those orally inoculated with FAdVs

Days post inoculation (DPI)	G1					G2					G3					G4				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
2		1	1				1	1				1				50	0	0	0	0
3			1					2				1				50	0	0	0	0
4		1	1	1	1		1	2	1	1		2	1	1		50	0	0	0	0
5		1			1		2			1					1	50	0	0	0	0
6				1	1				2	1						50	0	0	0	0
7												1				50	0	0	0	0
8												1				50	0	0	0	0
9		1					1									50	0	0	0	0
10								1								50	0	0	0	0
15		1						1								50	0	0	0	0
25		1					2					1	1			50	0	0	0	0
30			1					1				1				50	0	0	0	0
35		1					1					1				50	0	0	0	0

G1: Orally inoculated at one day of age with FAdVs type D.2 (MT386509.1); G2: Orally inoculated at one day of age with FAdVs type D.11 (MW847902); G3: Orally inoculated at one day of age with FAdVs type E (MW847902); G4: Negative non inoculated blank.

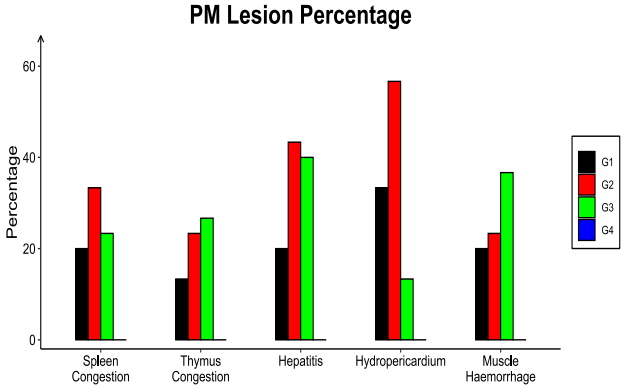

Fig. 3: Postmortem changes in all groups were detected and scored. G1, G2 and G3 were orally infected with FAdVs type D (MT386509.1, MT893206.1) and FAdVs type E (MW847902) respectively. G4 served as a blank control group.

Table 2: Number of dead birds at different DPI in control broiler chicks and those orally inoculated with FAdVs

DPI	G1	G2	G3	G4
3	1	1	0	0
5	1	2	0	0
7	1	1	1	0
17	1	1	0	0
31	0	1	0	0
32	1	1	0	0
33	1	1	1	0
35	1	0	1	0
Total	7	8	3	0

DPI: Days post inoculation; G1: Orally inoculated at one day of age with FAdVs type D.2 (MT386509.1). G2: Orally inoculated at one day of age with FAdVs type D.11 (MW847902). G3: Orally inoculated at one day of age with FAdVs type E (MW847902). G4: Negative non inoculated blank.

hepatocytes (Fig. 5c). At 5 DPI, histopathology of the liver in birds of G1 showed sinusoidal dilatation, moderate vacuolar degeneration, necrobiotic changes, and eosinophilic intranuclear inclusions surrounded by a halo in hepatocytes, mononuclear cells infiltration, and mild periportal fibrosis (Fig. 5d). The liver of chicks of G2 and G3 showed similar lesions to birds of G1 (Fig. 5e) but the leukocytes infiltration in G3 was less severe (Fig. 5f). At 7 DPI, microscopy of the liver in G1 chicken revealed moderate vacuolar degeneration of hepatocytes and mild multifocal leukocytes infiltration. In birds of G2 and G3, the liver lesions were similar to those observed in G1; however, the severity of lesions was mild in birds of G3. At 14 DPI, the liver of chicken showed mild vacuolar degeneration with mild multifocal mononuclear cells infiltration in G1 (Fig. 5g) and G2 (Fig. 5h). In chicks of G3, the liver showed periportal moderate multifocal mononuclear and heterophilic cells infiltration with mild fibrosis (Fig. 5i). At 21 DPI, the liver of chicken had mild vacuolar degeneration with mild multifocal mononuclear cells infiltration in G1 and G2, in addition to heterophils infiltration and fibrosis in G2. In birds of G3, the liver was infiltrated with mononuclear leukocytes and heterophils infiltration. At 28 DPI, the liver revealed moderate periportal mononuclear cells and heterophils infiltration in G1 (Fig. 5j) and G2 (Fig. 5k) and periportal mononuclear leukocytes infiltration with mild fibrosis in G3 (Fig. 5l). At 35 DPI, periportal leukocytic infiltration with mononuclear cells and heterophils and few intranuclear inclusion bodies (INIB) in mononuclear cells were observed in birds of G1. In G2, the liver showed well circumscribed vacuoles and mild vacuolar degeneration in hepatocytes. Moderate multifocal periportal mononuclear cells and heterophils infiltration and INIB were also observed. In G3, the liver exhibited changes similar to G1 but leukocytes infiltration and inclusion bodies were less common.

Histological examination of the kidney of chicken in the control group throughout the experiment revealed normal histological structures. At 3 DPI, the kidney of chicken in G1 revealed mild multifocal areas of peritubular mononuclear cells infiltration and mild degeneration of tubular epithelium. The glomeruli showed mesangial hyperplasia with thickening of Bowman's capsule and narrowing of Bowman's space which was consistent with proliferative glomerulonephritis. Inclusion bodies were poorly observed in the kidneys (Fig. 6a). In birds of G2, the kidney exhibited similar lesions to those of G1 but were more severe (Fig. 6b). In group G3, the kidney lesions were similar to those recorded in G1 and G2 but there were mild perivascular hemorrhages without leukocytes infiltration (Fig. 6c). At 5 DPI, kidney microscopy revealed glomerulonephritis, mild degeneration with large basophilic inclusion bodies in the tubular epithelium and mesangial cells, and mild

interstitial fibrosis in G1 (Fig. 6d); mild focal mononuclear leukocytes infiltration in G2 (Fig. 6e) and few peritubular leukocytes infiltration, mild degeneration of tubular epithelium, glomerulonephritis, and mild interstitial fibrosis in G3 (Fig. 6f). At 7 DPI, the kidneys had glomerulonephritis and few INIB in tubular epithelium in G1 and G2 and peritubular mononuclear leukocytes in G3 chicks. At 14 DPI, glomerulonephritis, glomerulosclerosis, mild focal mononuclear leukocytes infiltration, and mild tubular degeneration persisted in birds of G1, G2 and G3 (Fig. 6g). Large intranuclear eosinophilic inclusion body in Bowman's epithelium was seen in G2 (Fig. 6h). Glomerulosclerosis was less severe in G3 birds (Fig. 6i). At 21 DPI, glomerulonephritis, glomerulosclerosis, moderate focal mononuclear leukocytes infiltration, moderate necrobiotic changes in tubular epithelium, tubular casts, and eosinophilic to basophilic INIB were recorded in birds of G1, G2 and G3 but without leukocytes infiltration and IB in G2 and G3. At 28 DPI, renal lesions included multifocal mononuclear cells infiltration and focal areas of necrobiotic changes in the tubular epithelium in chicks of G1 (Fig. 6j). In G2, the renal lesions were almost regressed and were confined to a single focal mononuclear cells infiltration and mild glomerulonephritis (Fig. 6k). In G3, the kidneys still exhibited multifocal mononuclear leukocytes infiltration, glomerulonephritis and glomerulosclerosis (Fig. 6l).

Microscopic examination of the heart in the control group revealed normal histological structure throughout the study. At 3 DPI, birds of G1 showed mild focal myocardial vacuolation (Fig.7a and 2 Fig.7b). The cardiac blood vessels revealed vasculitis and thickening of wall with occasional mononuclear cell infiltration and hypertrophy of endothelial cells. In G3, the heart showed myocarditis, with moderate leukocytes infiltration and large basophilic intranuclear inclusions between the muscle bundles (Fig. 7c).

At 5 DPI, there was focal vacuolar degeneration in cardiomyocytes in birds of G1 (Fig. 7d), pericarditis, heterophilic cells infiltration between muscle fibers and vacuolar degeneration of cardiomyocytes in G2 (Fig. 7e), and degeneration of cardiomyocytes, heterophilic and mononuclear cells infiltration, and mild focal areas of fibrosis in G3 (Fig. 7f). At 7 DPI, there was heterophils infiltration between muscle bundles and focal mononuclear cells infiltration in chicks of G1, mild perivascular edema with occasional leukocyte infiltration in G2, and pericarditis with heterophilic cells infiltration in G3. At 14 DPI, edema, and mild diffuse mononuclear cells infiltration between the muscle bundles were seen in birds of G1 and G2 (Fig. 7g and Fig. 7h), while G3 birds showed vacuolar degeneration of cardiomyocytes, mild focal fibrosis, and pericarditis with mononuclear cells infiltration (Fig. 7i). At 21 DPI, there was edema, myocarditis, leukocytes (mononuclear and heterophils) infiltration, lysis of myocardiocytes, and vasculitis in chicks of G1 and G3, mild perivascular fibrosis, and fat cells in G2. At 28 DPI, the heart showed mild diffuse mononuclear cells infiltration and fibrosis in G1 (Fig. 7j), thickening and fibrinoid necrosis of the blood vessels, mild perivascular fibrosis and adipocytes infiltration in G2 (Fig. 7k), mild perivascular fibrosis, hemorrhage and occasional leukocytes infiltration in G3 (Fig. 7l). At 35 DPI, the heart showed fibrosis between muscle bundles in all inoculated groups.

Microscopy of the spleen of chicken in the control group exhibited normal histological structures throughout the experiment. At 3 DPI, spleen in the three inoculated groups revealed hyperplasia of reticular cells (multiple mitotic figures), with depletion of white pulp, occasional heterophils infiltration, and few large basophilic intranuclear inclusions in leukocytes. The splenic blood vessels showed hypertrophy of tunica media with narrowing of the blood vessel lumen (Fig. 8a-c). At 5 DPI,

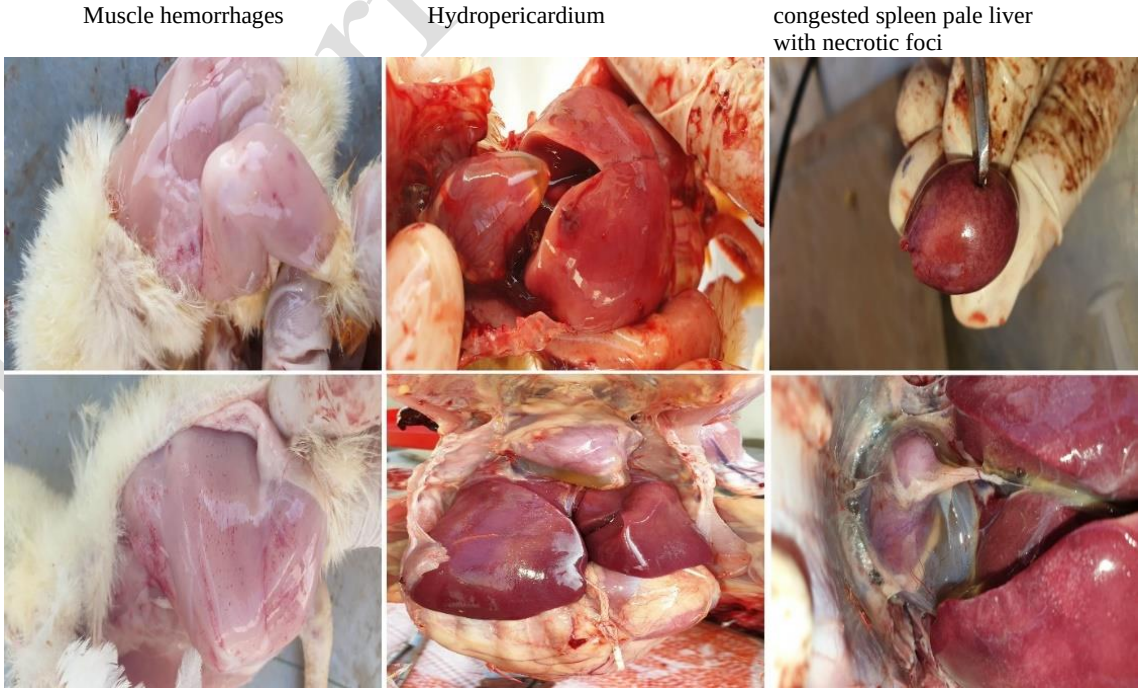

Fig. 4: Gross lesions of liver, spleen, heart and muscles. Macroscopic lesions in the FAdVs inoculated groups showing hydropericardium, enlarged pale liver with necrotic foci and hemorrhages on thigh muscles.

Table 4: Persistence of FAdVs by PCR in control broiler chicks and those experimentally inoculated orally with FAdVs

Sampling time	Cloacal swabs				Liver samples				Spleen samples			
	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
1 DPI	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2 DPI	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3 DPI	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
4 DPI	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve
5 DPI	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6 DPI	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7 DPI	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
14 DPI	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
21 DPI	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
28 DPI	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
35 DPI	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

- ve: Negative; +ve: Positive; DPI: Days post inoculation; G1: Orally inoculated at one day of age with FAdVs type D.2; (MT386509.1). G2: Orally inoculated at one day of age with FAdVs type D.11 (MW847902). G3: Orally inoculated at one day of age with FAdVs type E (MW847902). G4: Negative non inoculated blank.

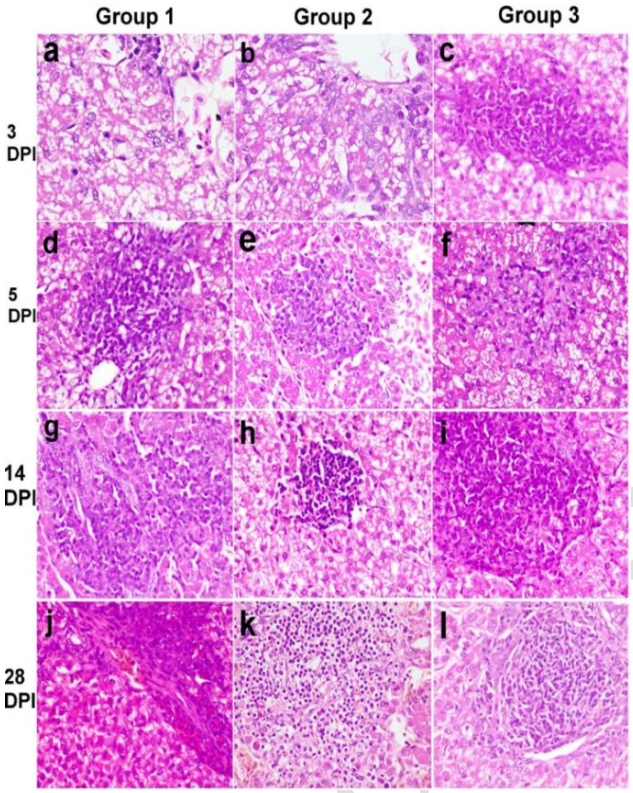

Fig. 5: Liver of chicken experimentally infected with FAdv. (a-b) 3 DPI, (a) vacuolar degeneration of hepatocytes in G1 and (b) G2 with intranuclear inclusions. (c) mononuclear cells infiltration in G3. (d-f) 5 DPI, (d) mononuclear cells (i).

the spleen showed severe depletion of white pulp and hyperplasia of reticular cells which sometimes had enlarged nucleus with intranuclear eosinophilic inclusion bodies. The blood vessels showed severe hypertrophy of tunica media and narrowing of lumen in birds of G1 (Fig. 8d). In G2 and G3, the spleen had similar lesions to G1, in addition to heterophilic cells infiltration in G2 and large basophilic INIB in lymphocytes in G3 (Fig. 8e-f). At 7 DPI, the spleen showed mild to moderate depletion of white pulp and reticular cells hyperplasia in birds of G1, G2, and G3. At 14 DPI, there was moderate lymphocytolysis in lymphoid follicles in G1 and G2 and mild lymphocytolysis in lymphoid follicles in G3 (Fig. 8g-i). At 21 DPI, moderate lymphocytolysis of lymphoid follicles was seen in chicks of G1 and G2 and moderate depletion of the white pulp in G3. At 28 DPI, there was lymphocytolysis in lymphoid follicles in G1, mild depletion of lymphoid follicles in G2, and few lymphocytolysis in lymphoid follicles in G3 (Fig. 8j-l). At 35 DPI, the spleen showed moderate depletion and infiltration of few heterophils in birds of G1, mild multifocal necrosis, bacterial aggregation, and moderate depletion of white pulp in G2, and moderate depletion of white pulp with large eosinophilic inclusions surrounded by a halo in lymphocytes in G3.

FAdVs were detected at 3 DPI in the liver, spleen and cloacal swab samples in birds of three inoculated groups. However, from 5 DPI no virus was detected in the liver or spleen of these birds. However, the virus was detected in the cloacal swabs till the end of the experiment in the three infected groups. No virus was detected in the control group (G4), as shown in Table 4 and Fig. 10.

Monitoring Concurrent Infection of CIAV and IBDV by PCR and RT-PCR

All groups were IBDV and CIAV negative throughout the experiment.

Serology

All the examined serum samples were negative as no precipitation lines were recorded in any sample by using AGPT (Fig. 11).

Influence of Experimental Infection of FAdVs on NDV HI Antibody Titers after Vaccination

Humoral immune response against NDV was almost similar in all groups on the day of vaccination and non-significance difference was observed among all groups at 1st-week post-vaccination. After two weeks post vaccination, HI titer was higher in the control group (G4) compared to the infected groups, with significant difference of NDV antibody titers between FAdVs infected groups and control one at 3rd-week post-vaccination (Table 3; Fig. 9).

Influence of Experimental Infection of FAdVs on Chicken Body Weight

The results revealed that all FAdVs infected groups had significantly lower ($P<0.05$) final body weight compared to the control group.

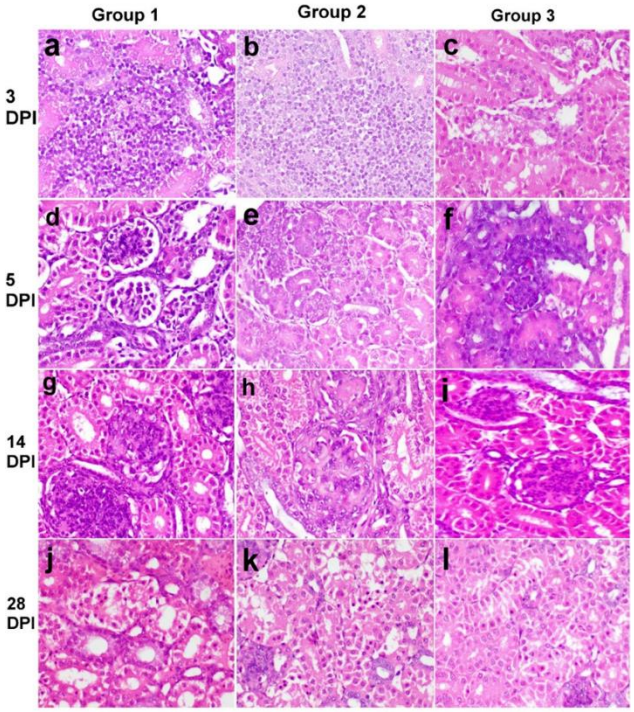

Fig. 6: Kidney of chicken experimentally infected with FAdv. (a-b) 3 DPI, (a) peritubular mononuclear cells infiltration in group 1 and (b) group 2. (c) Mild degeneration in the tubular epithelium in group 3. (d-f) 5 DPI, (d) glomerulonephritis with thickened glomerular basement membrane and large basophilic inclusion bodies in mesangial cells and tubular epithelium in group 1, (e) group 2, and (f) group 3. (g-i) 14 DPI, (g) glomerulosclerosis and tubular degeneration in group 1, (h) group 2 with eosinophilic INIB, and (i) group 3. (j-l) 28 DPI, (j) moderate necrobiotic changes in the tubular epithelium in group 1, (k) group 2, and (l) mild necrobiotic changes in group 3 (Hematoxylin and Eosin stain; X200).

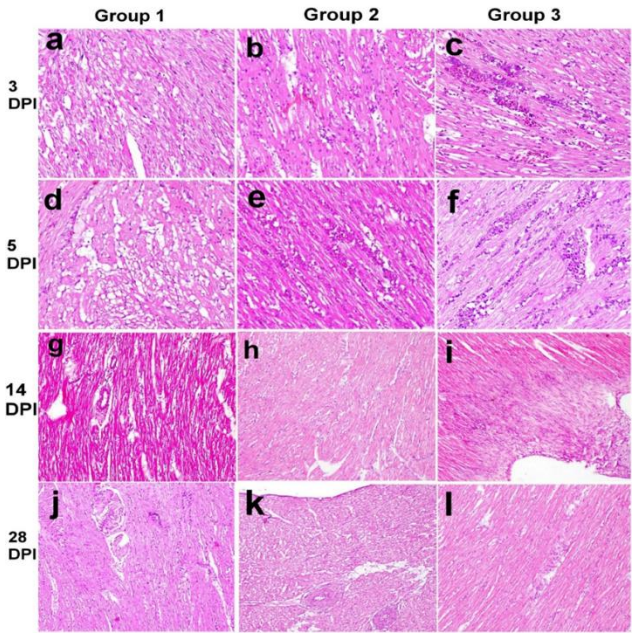

Fig. 7: Heart of chicken experimentally infected with FAdv. (a-b) 3 DPI, (a) mild focal myocardial vacuolation in G1 and (b) G2. (c) Moderate leukocytes infiltration with large basophilic intranuclear inclusions between the muscle bundles were observed in G3. (d-f) 5 DPI, (d) moderate focal vacuolar degeneration in cardiomyocytes in G1 and (e) heterophilic cells infiltration in G2, and (f) moderate leukocytes infiltration with large basophilic intranuclear inclusions in G3. (g-i) 14 DPI, (g) edema, and mild diffuse mononuclear cells infiltration between the muscle bundles in G1, (h) G2, and (i) mild focal fibrosis in group 3. (j-l) 28 DPI, (j) mild diffuse mononuclear cells infiltration and fibrosis in G1, (k) thickening and fibrinoid necrosis of the blood vessels, mild perivascular fibrosis and adipocytes infiltration in G2, and (l) mild perivascular fibrosis, hemorrhage and few leukocytes infiltration in G3 (Hematoxylin and Eosin stain; X200).

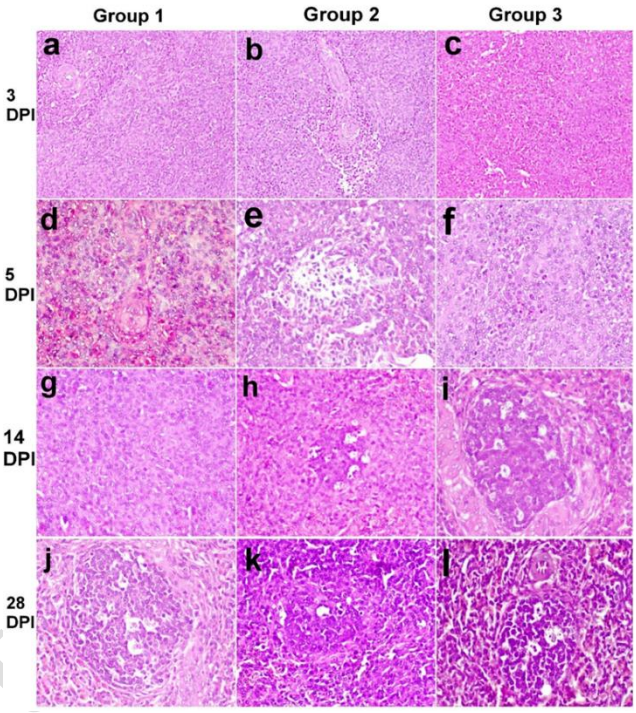

Fig. 8: Spleen of chicken experimentally infected with FAdv. (a-b) 3 DPI, (a) severe diffuse reticular cells hyperplasia and white pulp depletion in G1, (b) G2, and (c) group 3 (X100). (d-f) 5 DPI, (d) INIB in reticular cells (G1), (e) lymphocytes (G2), and (f) mitotic figures in reticular cells (G3). (g-i) 14 DPI, (g) severe reticular cells hyperplasia (G1), (h) lymphocytolysis in lymphoid follicle in G2, and (i) G3. (j-l) 28 DPI, (j) few lymphocytolysis in G1, (k) G2, and (l) G3 (Hematoxylin and Eosin stain; X200).

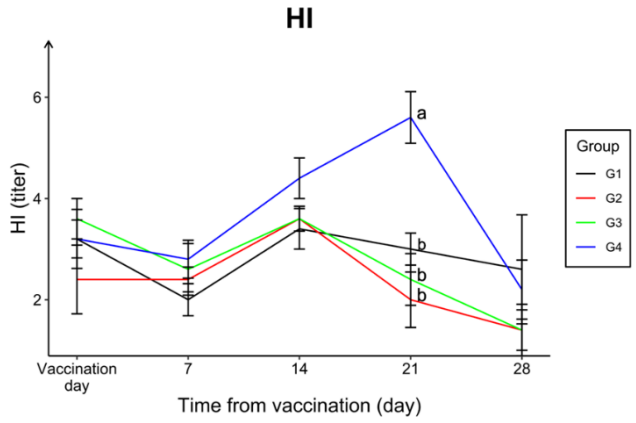

Fig. 9: HI titer against NDV in FAdVS infected and non-infected groups at different interval after NDV vaccination. G1, G2 and G3 were orally infected with FAdVs type D (MT386509.1, MT893206.1) and FAdVs type E (MW847902) respectively and G4 was left as a blank control group. Groups without sharing letters indicate significant differences between them at $P<0.05$.

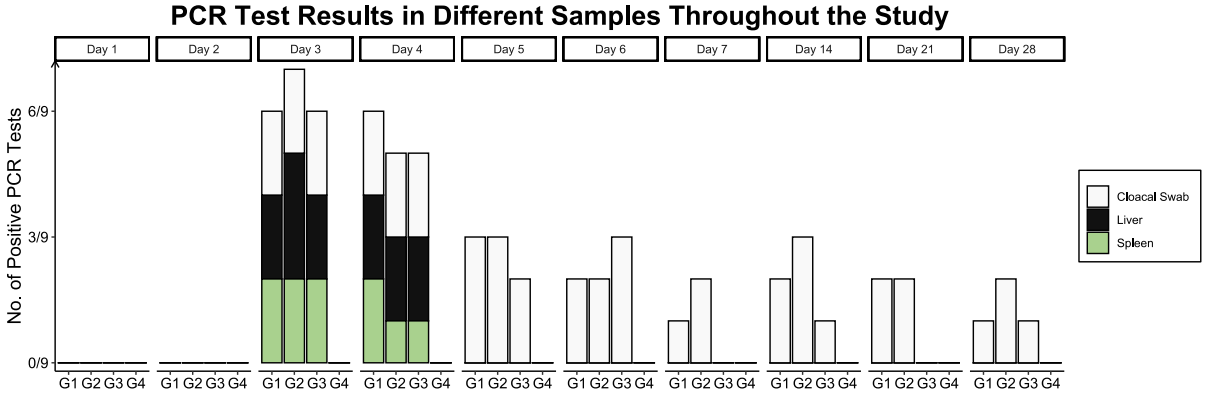

Fig. 10: Number of positive PCR tests of liver, spleen and cloacal swabs at different interval in all groups throughout the duration of the experiment. G1, G2 and G3 were orally infected with FAdVs type D (MT386509.1, MT893206.1) and FAdVs type E (MW847902), respectively and G4 was left as a blank control group.

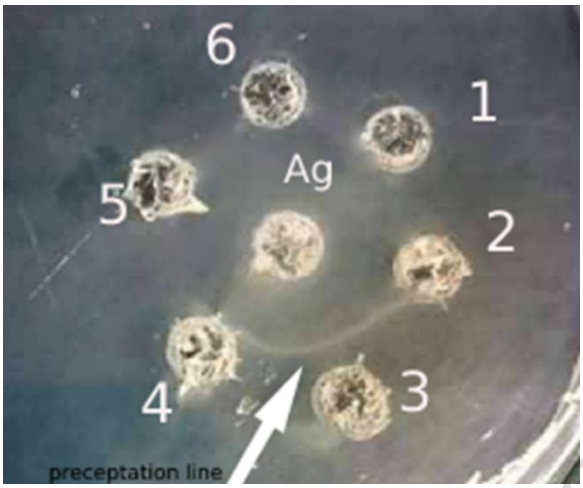

Fig. 11: Agar Gel Precipitation Test (AGPT): well No. 3 control FAdV positive: Arrows indicate precipitation line. All tested samples were negative (No precipitation lines).

DISCUSSION

During the past few years, FADVs were frequently reported globally, reflecting their widespread existence. The current study aimed to determine the variations in pathogenicity and virulence of FAdVs -2, FAdVs-11, and FAdVs-8a strains which were previously isolated from field cases of broilers chicken suffering from IBH/HPS (Al Naguib et al. 2021). It is well known that IBH may affect young chicks aged 3-6 weeks; however, early infection may occur during the first few days of life, possibly due to vertical transmission of infection (Steer et al. 2011). Therefore, one-day-old broiler chicks were used in the present study and the absence of vertical transmission was confirmed serologically by AGPT and negative PCR in different organs including liver, spleen and coloacal swabs. Experimental chicks were inoculated orally, as has been previously reported (Matos et al. 2016).

Clinical signs in the form of slight to severe depression, ruffled feathers, dropping wings and closed eyes were observed quite early from day 2 to 8 post inoculation in birds of G1, G2 and G3. These findings are supported by those previously described by Anjum et al. (1989) and Matos et al. (2016). Mortalities in all inoculated chicken groups were recorded and it was observed that chicks of G1, G2, and G3 showed higher death rate compared to chicks of G4 during the first-week post-infection; followed by a steady trend of mortalities during the 3rd and 4th weeks of infection. Then the death rate surged up at last week of the experiment. Neither clinical signs nor mortalities were recorded in the control group (G4) till the end of the experiment.

The most frequent pathological findings in both dead and euthanized birds included hydropericardium, pale liver with necrotic foci and subcapsular hemorrhages, enlarged and congested spleen, and petechial hemorrhages in breast and thigh muscles. Similar findings have been reported earlier by Thakor et al. (2012). Hepatitis was observed quite early at 2 DPI with the severity of gross lesions increased from 3 DPI to 7 DPI. However, hydropericardium and affections of the liver and spleen were seen throughout the experiment. Hydropericardium was severe in chicks of G1 and G2 compared to G3, while hepatic damage was more prominent in G3 than G1 and G2 chicks.

Degenerative changes and modest periportal mononuclear inflammation were observed in the liver of the orally infected birds, starting at 3 DPI; the degenerative changes lasted until 14 DPI, when they were reversed. These results are similar to prior observations recorded by Steer et al. (2015) and Ren et al. (2019). Nevertheless, periportal inflammation persisted and heterophils were observed in the periportal area at the end of the experiment, suggesting the occurrence of secondary bacterial infection (Steer et al. 2015).

The glomerulonephritis induced by FAdVs was more severe in chicken inoculated with FAdV-2 and FAdV-11 serotypes compared to FAdV-E 8a serotype. Similar findings were reported earlier by other studies (Casimiro et al. 2010; Mariappan et al. 2018). Glomerulosclerosis and INIB were observed in the kidney at 14 DPI. Although these lesions were regressed, they could be be seen till end of the experiment. Pericarditis and myocarditis with heterophils infiltration were prominent in FAdVs-E 8a infection at 3 DPI and regressed at 14 DPI, when fibrosis dominated in these lesions. FAdV-2 and FAdV-11, on the other hand, caused mild degenerative changes and inflammation in the heart. According to Schachner et al. (2018), FAdV can induce

Table 3: HI mean titer against NDV at different DPV in control broiler chicks and those inoculated with FAdVs and vaccinated with NDV vaccines.

Days post vaccination (DPV)	HI Titre (log2)			
	Group 1	Group 2	Group 3	Group 4
0	3.2±0.41833	2.4±0.875595	3.6±0.421637	3.2±0.65192
7	2.0±0.447214	2.4±0.316228	2.6±0.632456	2.8±0.447214
14	3.4±0.433861	3.6±0.258199	3.6±0.258199	4.4±0.381385
21	3.0±0.365148a	2.6±0.744208a	2.4±0.658281a	5.6±0.430946b
28	2.6±1.335895	1.4±0.861892	1.4±0.676123	2.2±0.786245

Groups without sharing letters indicate a significant (P<0.05) difference between them. DPV: Day post vaccination; Birds were vaccinated with NDV vaccines at 9 days of age with inactivated vaccine via *S/C injection* rout, at 10 days with *NDV HB1* via eye drop route and 18 days with *NDV Lasota* via eye drop route. DPI: Days post inoculation; G1: Orally inoculated at one day of age with FAdVs type D.2 (MT386509.1). G2: Orally inoculated at one day of age with FAdVs type D.11; (MW847902). G3: Orally inoculated at one day of age with FAdVs type E (MW847902). G4: Negative non inoculated blank.

degenerative and inflammatory changes in organs other than the liver, mainly in the heart, kidneys, lungs, and intestine.

In birds of different inoculated groups, spleen showed diffuse reticular hyperplasia and severe depletion of the white pulp. Similarly, Steer et al. (2015) reported diffuse lymphoid depletion in the white pulp with spindloid to polygonal cells having abundant eosinophilic cytoplasm surrounding the sheathed capillaries in the spleen of one-day-old chicks inoculated with field strains of fowl adenovirus serotypes 1, 8b and 11.In the present study, these lesions were partially alleviated at 14DPI, however, lymphocytolysis was observed in lymphoid follicles of different groups till the end of the experiment, which is in accordance with the results of a chronological study performed earlier (Steer et al. 2011).

Previously, Pallister et al. (1996) and Zhao et al. (2015) have described in detail the molecular pathogenesis of FAdVs isolates, including nucleotide sequence and expression analysis of the hexon and fibre genes that may explain the variations in disease severity observed between FAdV-D and FAdV-E 8a serotypes inoculated groups. Several PCR techniques for detection of FAdVs have been published and PCR for the detection of FAdVs is now well established (Raue et al. 1998; Steer et al. 2011; Asthana et al. 2013; Hess 2000; Cui et al. 2020).

The hexon gene has been employed for primer design in the bulk of published PCR approaches for detecting avian adenoviruses (Raue et al. 1998; Günes et al. 2012). In our study, FAdVs were detected quite early (3 DPI) in the liver, spleen and cloacal swab samples in birds of G1, G2, and G3. However, no virus could be detected in the liver or spleen of chicks of these three groups at 5 DPI. These findings are in agreement with those recorded in a previous study (Hafez 2011). On the other hand, the virus was still detected in the cloacal swabs till the end of the experiment in the three infected groups, which is supported by similar findings recorded by Radwan et al. (2019). No virus was detected in the control group (G4) at any stage.

Neither CIA nor IBDV was detected in experimentally infected birds showing symptoms of IBH/HP in the present study, indicating that Adenoviruses are the primary pathogen and are able to induce clinical signs and lesions in the absence of other immunosuppressive agents. However, these results disagree with some earlier studies where the concurrent infections of CIA or IBDV with FAdVs were recorded (Schachner et al. 2018; Elbestawy et al. 2020).

Significance decrease in antibody titers against NDV vaccine in the birds of three FAdVs infected groups compared to the control group indicates that FAdV-2, 11 and 8a strains may play a role as immunosuppressive pathogens that may result in vaccination failure in chickens in Egypt. Likewise, Saifuddin et al. (1992) and Schonewille et al. (2008) made similar suggestions previously.

In the present study, the final body weight of chicks of FAdVs inoculated groups was significantly decreased compared to the control birds, although all birds were reared under the same conditions and they consumed almost the same amount of feed during the study. Understanding the pathogenesis of FAdVs may explain this decrease in the body weight of infected chicks. According to Matos et al. (2016), FAdVs replication in liver and pancreas results in metabolic disorders, causing significant changes in clinical chemistry analytes including total proteins, albumin, AST, GLDH, bile acids, uric acid and lipase measured in the plasma of infected birds which confirmed tissue damage and functional impairment of both liver and pancreas.

Based on the results of clinical manifestation scoring, gross and microscopic changes, mortality rates and the final body weights, it appears that effects on all parameters were slightly more severe in the FAdV-D inoculated groups compared to FAdV-E 8a inoculated group. This suggests that FAdVs-D isolates are more virulent than FAdVs-E 8a isolates.

Conclusion

To our knowledge, this is the first study in which the pathogenicity of locally isolated Adenovirus type D and Type E in broilers chicks was investigated in Egypt. This study confirmed the ability of Adenovirus to induce clinical signs, gross and microscopic lesions of IBH/HP as a primary pathogen without concurrent infection with other immunosuppressive pathogens. Further studies may be conducted to investigate the immunosuppressive effect of these isolates in broiler chicken.

Availability of Data and Materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Competing Interests

The authors declare that they do not have any competing interests.

Funding

This research article received no specific grant from any funding agency.

Authors' contributions

GAA, MGN and EAM designed the study; MGN performed isolation and titration of viruses; DGK provided scientific input for the study; MGN wrote the manuscript; MSK provided histopathological data; GAA, MGN and EAM conducted the animal experiments; GAA, DGK and EAM supervised the study and reviewed the final manuscript.

Acknowledgements

We thank Dr. Mahmoud Gamal (Assistant Lecturer at Faculty of Veterinary Medicine) for assistance with statistical analysis of data.

REFERENCES

Al Naguib MG, Morsy EA, Samir M, Khattab MS, Khelfa DG and Abdel-Alim GA, 2021. Isolation and molecular characterization of fowl Adenoviruses associated with inclusion body hepatitis-hydropericardium syndrome in broiler chickens in Egypt. Veterinary World 11: 333-342. https://doi.org/10.54203/scil.2021.wvj46

Anjum AD, Sabri MA and Iqbal Z, 1989. Hydropericarditis syndrome in broiler chickens in Pakistan. The Veterinary Record 124: 247-248. https://doi.org/10.1136/vr.124.10.247

Asthana M, Chandra R and Kumar R, 2013. Hydropericardium syndrome: Current state and future developments. Archeive Virology 158: 921-931. https://doi.org/10.1007/s00705-012-1570-x

Casimiro DR, Cox K, Tang A, Sykes KJ, Feng M, Wang F and. Flynn J, 2010. Efficacy of multivalent Adenovirus-based vaccine against Simian immunodeficiency virus challenge. Journal of Virology 84: 2996-3003.

Chaudhry KM, Chaudhry HR, Chaudhry M and Chaudhry AM, 2012. A Manual of Diagnostic Procedures for Viral Diseases of Poultry (In Poor Resourse Setting-Revised). CreateSpace Independent Publishing Platform South Carolina, USA.

Cook JK, 1983. Fowl adenoviruses: Studies on aspects of the pathogenicity of six strains for 1-day-old chicks. Avian Pathology 12: 35-43.

Cui J, Xu Y, Zhou Z, Xu Q, Wang J, Xiao Y, Li Z and Bi D., 2020. Pathogenicity and molecular typing of fowl adenovirus-associated with hepatitis/hydropericardium syndrome in central China (2015–2018). Frontiers in Veterinary Science 7: 190. https://doi.org/10.3389/fvets.2020.00190

Del Valle FP, Camba SI, Umali DV, Sasai K, Shirota K, Katoh H and Tajima T, 2020. Research note: Molecular and pathologic characterization of avian adenovirus isolated from the oviducts of laying hens in eastern Japan. Poultry Science 99: 2459-2468.

Elbestawy AR, Ibrahim M, Hammam H, Noreldin A, Bahrawy A and Ellakany HF, 2020. Molecular characterization of fowl Adenovirus D species in broiler chickens with inclusion body hepatitis in Egypt. Alexandria Journal of Veterinary Sciences 64: 110-117. https://doi.org/10.5455/ajvs.74411

Günes A, Marek A, Grafl B, Berger E and Hess M, 2012. Real-time PCR assay for universal detection and quantitation of all five species of fowl adenoviruses (FAdV-A to FAdV-E). Journal of Virological Methods 183: 147-153. https://doi.org/10.1016/j.jviromet.2012.04.005

Gupta A, 2018. Pathogenesis and control of inclusion body hepatitis in broiler chickens. PhD Thesis, University of Saskatchewan, Saskatoon, Canada.

Hafez HM, 2011. Avian adenoviruses infections with special attention to inclusion body hepatitis/hydropericardium syndrome and egg drop syndrome. Pakistan Veterinary Journal 31: 85-92. http://dx.doi.org/10.17169/refubium-18486

Hess M, 2000. Detection and differentiation of avian adenoviruses: A review. Avian Pathology 29: 195-206. https://doi.org/10.1080/03079450050045440

Jones R and Georgiou KJAP, 1984. Experimental infection of chickens with an adenovirus isolated from tenosynovitis, following infection with either (1) an arthrotropic reovirus, or (2) infectious bursal disease virus. Avian Pathology 13: 289-302.

Mariappan AK, Munusamy P, Latheef SK, Singh SD and Dhama K, 2018. Hepato nephropathology associated with inclusion body hepatitis complicated with citrinin mycotoxicosis in a broiler farm. Veterinary World 11: 112.

Matos M, Grafl B, Liebhart D and Hess M, 2016. The outcome of experimentally induced inclusion body hepatitis (IBH) by fowl aviadenoviruses (FAdVs) is crucially influenced by the genetic background of the host. Veterinary Research 47: 69. https://doi.org/10.1186/s13567-016-0350-0

McFerran J and Smyth J, 2000. Avian adenoviruses. Revue Scientifique et Technique (International Office of Epizootics) 19: 589-601. doi:PMID: 10935281.

McFerran JB and Adair BM, 1977. Avian adenoviruses: A review. Avian Pathology 6: 189-217. https://doi.org/10.1080/03079457708418228

Pallister J, Wright PJ and Sheppard MJ, 1996. A single gene encoding the fiber is responsible for variations in virulence in the fowl adenoviruses. Journal of Virology 70: 5115-5122. https://doi.org/10.1128/jvi.70.8.5115-5122.1996

Radwan MM, El-Deeb AH, Mousa MR, El-Sanousi AA and Shalaby MA, 2019. First report of fowl adenovirus 8a from commercial broiler chickens in Egypt: Molecular characterization and pathogenicity. Poultry Science 98: 97-104. https://doi.org/10.3382/ps/pey314

Raue R and Hess M, 1998. Hexon based PCRs combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenoviruses and egg drop syndrome virus. Journal of Virological Methods 73: 211-217. https://doi.org/10.1016/S0166-0934(98)00065-2

Ren G, Wang H, Yan Y, Liu F, Huang M and Chen R, 2019. Pathogenicity of a fowl adenovirus serotype 4 isolated from chickens associated with hydropericardium-hepatitis syndrome in China. Poultry Science 98: 2765-2771. https://doi.org/10.3382/ps/pez042

Saifuddin M and Wilks C, 1991. Pathogenesis of an acute viral hepatitis: inclusion body hepatitis in the chicken. Archives of Virology 116: 33-43.

Saifuddin M and Wilks C, 1992. Effects of fowl adenovirus infection on the immune system of chickens. Journal of Comparative Pathology 107: 285-294. https://doi.org/10.1016/0021-9975(92)90004-E

Schachner A, Grafl B and Hess M, 2021. Spotlight on avian pathology: Fowl adenovirus (FAdV) in chickens and beyond–an unresolved host-pathogen interplay. Avian Pathology 50: 2-5. https://doi.org/10.1080/03079457.2020.1810629

Schachner A, Matos M, Grafl B and Hess M, 2018. Fowl adenovirus-induced diseases and strategies for their control–a review on the current global situation. Avian Pathology 47: 111-126. https://doi.org/10.1080/03079457.2017.1385724

Schonewille E, Singh A, Göbel TW, Gerner W, Saalmüller A and Hess M, 2008. Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid organs in

specific pathogen-free chickens following experimental infection. Veterinary Immunology and Immunopathology 121: 130-139.

Sohaimi NM and Clifford UC, 2021. Fowl adenovirus in chickens: Diseases, epidemiology, impact, and control strategies to the Malaysian poultry industry–A review. Journal of World's Poultry Research 11: 387-396.

Steer PA, O'Rourke D, Ghorashi SA and Noormohammadi AH, 2011. Application of high-resolution melting curve analysis for typing of fowl adenoviruses in field cases of inclusion body hepatitis. Australian Veterinary Journal 89: 184-192. https://doi.org/10.1111/j.1751-0813.2011.00695.x

Steer PA, Sandy JR, O'Rourke D, Scott PC, Browning GF and Noormohammadi AH, 2015. Chronological analysis of gross and histological lesions induced by field strains of fowl adenovirus serotypes 1, 8b and 11 in one-day-old chickens. Avian Pathology 44: 106-113. https://doi.org/10.1080/03079457.2015.1007919

Thakor KB, Dave CJ, Fefar DT, Jivani BM and Prajapati KS, 2012. Pathological and molecular diagnosis of naturally occurring inclusion body hepatitis-hydropericardium syndrome in broiler chickens. Indian Journal of Veterinary Pathology 36: 212-216.

Toro H, Gonzalez O, Escobar C, Cerda L, Morales M and Gonzalez C, 2001. Vertical induction of the inclusion body hepatitis/hydropericardium syndrome with fowl adenovirus and chicken anemia virus. Avian Diseases 45: 215-222. https://doi.org/10.2307/1593031

Woernle H, 1966. The use of the agar-gel diffusion technique in the identification of certain avian virus diseases. The Veterinarian Journal 4: 17-28.

Zhao J, Zhong Q, Zhao Y, Hu YX and Zhang GZ, 2015. Pathogenicity and complete genome characterization of fowl Adenoviruses isolated from chickens associated with inclusion body hepatitis and hydropericardium syndrome in China. PLoS One 10: e0133073. https://doi.org/10.1371/journal.pone.0133073