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Original Research Article

Fowl Adenovirus 8b (FAdV-8b) Group E Causes hepatitis Hydropericardium Syndrome (HHS) In Broiler Chickens

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Conflict of interest: Nil

Abstract:

Fowl adenovirus (FAdV) infects chickens and leads to hepatitis, in a condition called inclusion body hepatitis (IBH), and hepatitis with accumulation of fluids in the pericardial sac of the heart in a condition known as hepatitis hydropericardium syndrome (HHS). Both conditions are associated with other pathologies in several organs in addition to these stated features. However, the fluid in pericardial sac distinguishes HHS from IBH. IBH is caused by Fowl adenoviruses group D, serotype 2-11, and group E, serotypes 8a, 8b and 11. The adenoviruses that cause HHS is of group C, serotype 4. In this study, the postmortem and pathologic findings found characteristic hepatitis and hydro pericardium of HHS. Hepatitis and hydropericardium were confirmed grossly, histologically and biochemically by testing ALT and AST in infected chickens. Deformity In the heart, renal and hepatic hemorrhages, necrosis in liver, kidney and heart muscle, and expansion of the pericardial sac, inclusion body in hepatocytes are reported in this study. The PCR, Hexone gene sequence and phylogenic tree confirmed the involvement of adenovirus group E in HHS. This finding was reported for the first time. The discovery that HHS is caused by group E will have implications for vaccine usage in the field.

Keywords: HHS, IBH, Fowl Adenovirus, Hepatitis, Jordan, Vaccine.

Introduction

The Fowl adenoviruses (FadV) are double-stranded DNA of 70–100 nm in diameter and belong to genus Aviadenovirus of Adenoviridae family [1]. Fowl adenovirus is composed of five species (A–E) and 12 serotypes based on the conserved Hexon gene; hexon and fiber are the two major structural proteins [2]. IBH predominantly impacts birds aged 3 to 7 weeks, and its consequences range from 5% to 30% mortality rate Influencing factors that include Virulence and the use of immunosuppressive agents [3]. The FAdV-8b can be passed both from breeders to their offspring as well as through fecal-oral contact between the birds enhancing dissemination in the flock. Aviadenovirus cause several diseases including inclusion body hepatitis (IBH), hepatitis hydropericardium syndrome (HHS), adenoviral gizzard erosion (AGE), avian adenoviral splenomegaly (AAS) and egg drop syndrome (EDS) [4]. Fowl adenoviruses blamed for IBH belong to species D, serotype 2-11 , and group E, serotypes 8a, 8b and 11. Fowl adenoviruses responsible for HHS belong to group C, serotype 4 and group C viruses, serotype 1 are responsible for gizzard erosion disease [4]. while HHS is caused by adenovirus of species C (serotype 4), and gizzard erosion is caused by Species A strain FAdV-1 [5]. Interestingly, there is no cross protection between serotypes and this may be due to the non-identical genome structure of adenoviruses [6]. Inclusion body hepatitis (IBH) is a disease of broilers in the age of 3- to 7-week-old. but younger birds and older birds of 20 weeks of age have been infected [7]. It was reported in many avian species, including turkeys, pigeons, geese, and psittacine birds in different parts of the world [8]. The Pulmonary manifestation in IBH due to FAdV, FAdV-8b of group E, is high, and the mortality rates among affected birds have ranged between 2% to a high of 40% [9]. Fowl adenovirus serotype 4 (FAV4) causes a specific form of the disease in broilers characterized by accumulation of yellow fluid in the pericardial sac, in a disease called infectious hydropericardiumor hepatitis/hydropericardium syndrome [10]. It infects broilers at 3-5 weeks of age. Adenovirus has been isolated from several organs and tissues previously. For example, Adenovirus group 1 was isolated from feces, gizzard, hydropericardium and liver and group 2 from spleen. These viral groups

belong to serotype 1, 2, 8 and 4 [11]. Co-infections (mixed) with various adenovirus serotypes in the same bird was confirmed [12, 13], also co-infection with other viruses such as infectious bronchitis virus (IBV), reoviruses, and Newcastle disease virus (NDV) was also observed [13]. Some studies suggested that immunosuppressive infections must occur before adenovirus clinical signs develop. This note has been refuted recently [14]. However, FAdV suppress the immune system by itself without the need for other immunosuppressive agents [15]. In addition to co-infections, the severity of the disease depends on the age of birds, immune status and pathogenicity of the virus [9]. The virulence of FADVs is a complex issue as there is no specific or single genomic feature identified as genetic virulent factor. The circulating adenoviruses recombine and thus produce less or more virulent strain, and create more FAdV with unknown genetic traits. The genetic deletion in FAdV is another source of differences. However, sequence data show no distinct differences between hexon and fiber genes of pathogenic and nonpathogenic FAdV [16].

The infectious diseases caused by FAdV infections in avian species in Jordan such as IBH and HHS, was not identified and the current situation of adenovirus is not known, in spite of the high mortality and economic losses. HHS is a severe syndrome that is characterized by accumulation of clear, yellow or gelatinous fluids in the pericardial sac and heart deformities. Lesions in the liver include multifocal necrosis, intranuclear inclusion bodies in hepatocytes and high mortalities [17]. Clinical symptoms included anorexia, depressed attitude, fluffed-up feathers, huddling, and green diarrhea. This include mortality which began at day eight of age and varied between 10% and 14%. At the post-mortem findings indicated severe hepatitis, jaundice, en pancreatitis, a liver which was pale, enlarged hemorrhagic and friable, kidneys and spleen swollen, hemorrhagic [18]. The incubation period isfrom 6 to 15 days in naturally infected chickens, and from 48 to 72 h, occasionally up to 7 days experimentally and the duration of the infection is generally reported as 2 or 3 weeks [19].

Fowl adenoviruses are readily transmitted horizontally since they are present in all excretions, and at high titers in the feces. Fomites, personnel, and transport can also be important contributors to the spread of the virus [20]. The viral shedding depends on administered dose of the virus and higher viral load in chickens increased the shedding rate of the virus mainly in feces [21]. The incubation period varied from 6 to 15 days in naturally infections and from 48 to 72 h, occasionally up to 7 days experimentally and the duration of the infection is generally reported as 2 or 3 weeks [19,20].The clinical signs of the FadV include pale swollen livers with hemorrhage and the presence of basophilic inclusion bodies in the hepatocytes. Inflammation and necrosis of the liver [21,22].The main postmortem finding in HPS are the accumulation of a clear, straw colored fluid in the pericardial sac and mortality rate of 40-60%.

The birds are observed to be dull, rolling with wings up, covered in fluffed poof yellow feces. Those having symptoms get lesions in the liver and the kidney. The kidneys are pale, swollen and have mottled appearance and hemorrhage into the renal cortex [23,24]. Microscopic lesions include basophilic intranuclear inclusion bodies in liver cells, and inflammatory leucocytes aggregates in some areas. The kidneys show congestion and focal hemorrhage, vaculations, the heart show edema, muscle degeneration, necrosis, and mononuclear infiltration [10,23,25]. The purpose of the present study is to understand the prevalence, histopathology, and routes through which Fowl adenovirus serotype 8b (FAdV-8b) spreads in the broiler chickens. The objectives are to estimate the prevalence and genetic variations of FAdV-8b within the targeted population of affected poultry, to describe clinically observed sign, mortality rates, and histological alterations in connection with FAdV-8b, to outline the effects of co-infections, immunosuppression and environmental effects on FAdV-8b infections in broiler poultry.

Materials and Methods

Sample collection

Postmortem examination was carried out on the affected farm on multiple birds. The internal organs were exposed and examined grossly for pathological lesions. Samples from organs for histological examination were collected and kept in 10% formaldehyde. Chickens were also collected from another distant farm that showed no signs of any disease and were subjected to postmortem examination and organs were taken for microscopic examination.

Sample staining with routine stain

At necropsy, a small piece from the liver, kidney and heart was collected and kept in 10% formalin for 72 hours. The samples were then dehydrated by passing them in ascending concentration of ethanol (50, 60, 70, 60, 90 and 100%) for one hour consequently. The samples were then placed in xylene for one hour and then in liquid paraffin for 2 hours. The samples were placed in special molds, and liquid paraffin was poured into the tissues. The samples were left at room temperature for half an hour and placed in a fridge for 10 minutes before sectioning. They were cut at 5 micrometre thickness using a rotary microtome. The samples were then stained in hematoxylin and eosin stains, hydrated into ascending concentration of ethanol,

mounted by Canada balsam, and covered by a glass cover slip. The slides were viewed, and images were captured by a camera connected to a light microscope.

PAS stain

The slides were stained with Periodic Acid Schiff stain after normal procedure. Firstly, the slides were washed by hydration and oxidation in 0.5% periodic acid half an hour. They were then washed three time with distilled water. Similarly, the third set of slides was stained in Schiff's reagent for 15 minutes then washed in tap water for 5 minutes. Thereafter, they were stained with hematoxylin for 1 minute, washed with distilled water, rehydrated and covered with DPX. The results were analyzed by using light microscopy at 40X magnification. In the end, the obtained slides were visualized under the light microscope and the images are captured using a camera mounted on it.

ALT and AST levels in the liver

Aspartate transaminase (AST): in a test tube, the enzyme substrate was incubated for 5 minutes at 37°C and then 200 µl serum sample was added, and the mixture was incubated at 37°C for 60 minutes. Then 1 ml of a reagent consisting of 2,4-dinitrophenyl-hydrazine (DNPH) 1mmol/ and HCL 1mol/l was added to the tube and mixed and incubated for 20 minutes at room temperature. This step was followed by adding and mixing 10ml of 0.4N sodium hydroxide which was incubated for 5 minutes. The absorbance was read at 505 nm. The results were obtained from a standard curve supplemented by the kit manufacturer (biolabo, France) and the OD was obtained by spectrophotometer at 340 nm. The concentration of AST was calculated using the formula:

U/L=1746x delta 340nm/min

Alanine aminotransferase (ALT)

All reagents and samples were brought to room temperature. 1 ml of a mixture consisting of 15mmol/l 2-oxoglutarate, 500mmol/l L-alanine, 1600IU LDH, less than 0.18mmol/l tris buffer with a ph. adjusted to 7.5 at 30°C was added to 100 µl serum sample. The absorbance was measured every 1 minute for three times according to the kit manufacturer (Biosystems S. A. Spain). The concentration of ALT was calculated according to the formula

$$\text{Activity (U/L)} = \Delta\text{Abs/min} \times \text{factor}$$

$$\text{Factor} = \frac{\text{VR} \times 1000}{6.3 \times \text{VE} \times \text{P}}$$

VR symbolises total reaction volume in mL, VE signify the specimen volume in mL, 6.3 is the molar extinction coefficient of NADH at 340 nm and P refers path length employed while spectrophotometric assays.

DNA Extraction and amplification

The tested samples taken from broiler chickens organs and tissues (liver 8 samples, heart 3 samples, kidney 1 sample, blood 4 samples and 1 pericardial fluid sample).

DNA extraction was carried out using the DNA Mini Kit for animal tissue (Qiagen GMbH, Hilden, Germany) according to the manufacturer's direction. DNA was prepared from organ samples (25 mg), blood (200 µl), or pericardial fluid (200 µl) by lysing in 180 µl of ATL buffer containing 20 µl of proteinase K at 56°C for 3 hours. In the next step, lysis was done and 200 µl of buffer AL was pipetted to the mixture and the contents were incubated at 70°C for 10 minutes. The lysate was then applied on QIAamp Mini Spin Column, washed twice with 500µl AW1 and AW2 buffers and finally eluted in 100µl AE buffer.

DNA purity was measured using the absorbance spectroscopy by Nanodrop spectrophotometer (Thermo Scientific, USA). The amplification of hexon gene using Multiplex PCR was done using the forward primer HexF1 (5'-GAYRGYHGGRTNBTGGAYATGGG-3') and reverse primer HeXR1 (5'-TACTTATCNACRGCYTGRTTCCA-3') [8]. Standard PCR reaction mixture was prepared to contain an 10µl final volume including 3µl of DNA, 2µl of master mix, 0.3µl of each hexapeptide nucleotide (HexF1 and HeXR1), and 4.7µl of nuclease-free water. PCR cycling comprised of 35 cycles of 94°C for 30 seconds and 72°C for 30 seconds which yielded an amplicon of about 800 bp as determined by PCR product standards Molecular weight markers were run concurrently on a 2% agarose gel stained with Ethidium Bromide then visualized on a UV light.

Results

Postmortem findings

Postmortem examination showed friable necrotic liver with areas of severe congestion. Severe bleeding upon dissecting the liver. Some livers were pale. The kidneys were hemorrhagic and inflamed. Marked accumulation of yellowish colored fluids around the heart filling the pericardial space and hemorrhages were evident on the breast and thigh muscles. Congested intestine and greenish diarrhea were seen. The mortality rate was 13.3%**.** Other signs include the yellow color of internal fat and dermis. Heart deformity, fluid-filled pericardial sac, green diarrhea in some birds. The findings are shown in (Figure 1a-g).

Pathological findings

Microscopic examination revealed that the liver was congested, hemorrhagic, with distinctive fatty changes and the presence of typical inclusion bodies in hepatocytes which are compatible with the diagnosis of inclusion body hepatitis. The heart showed expansion of pericardial space which was filled with fibrinous and cellular contents and cardiomyocytes necrosis. The kidneys showed renal tubular necrosis, and tubular separation from the underlining basement membrane and irregular basement membrane, (Figure 2 a-f)

Clinical Chemistry Data

Liver homogenate of adenovirus infected chickens had majorly higher ALT and AST levels of 7 ± 0.67 U/L and 2470.6 ± 574.6 U/L than that of the control group. Chickens that were not infected had a lower mean ALT and mean AST concentration than the infected birds of 0.07 ± 0.036 U/L and 32.21 ± 7.77 U/L, respectively. ALT activities of adenovirus infected chickens were 10- fold higher than that of the non-infected chickens while AST activities were 317 folds higher compared to the non-infected chickens. These elevated values observed in the infected chickens suggest virulent liver pathology linked with HHS adenovirus infection as presented in the figures 3a and 3b.

Viral Isolation by PCR

All the 16 samples obtained from the infected chickens were PCR positive for adenovirus, and DNA products of about 800 bp were observed in the agarose gel. Positive results were also confirmed in liver, heart, pericardial, blood, and renal tissues (Figure 4a). On the other hand no viral DNA was seen in the PCR samples from non-infected chickens (Figure 4b). These confirmatory results demonstrate the successful identification of the hexon gene that is characteristic of fowl adenovirus in samples from infected chickens, although there were no positive control results for non-fowl adenovirus samples.

Sequence and phylogenetic analysis

The sequencing of hexon gene from the isolates from chicken organs and tissues (liver, kidney, heart, pericardial fluid and blood) was analyzed. The isolates were Fowl aviadenovirus E strain FAdV-E hexon gene, partial cds (accession number MN052902.1). The isolated virus strains in the current study (marked by) are identical, which means that chickens were infected with the same viral strain. The analysis of the sequence of this isolate was carried out by Blast search (https: //blast.ncbi.nlm.nih.gov) and phylogenic tree construction. The detected field isolates were found to be related to FAdV-8b (species E) with > 99.82% sequence identity and they are highly related to these four strains (IS4346/2021 hexon gene, partial cds, TR/BVKE/CA/CAK hexon gene, partial cds, TR/BVKE/R/D-1 hexon gene, partial cds and TR/BVKE/CA/CYG hexon gene, partial cds). The accession numbers of these strains in GenBank database are MZ368700.1, MK937073.1, MK937075.1, MK937074.1 respectively as shown in figure 5.

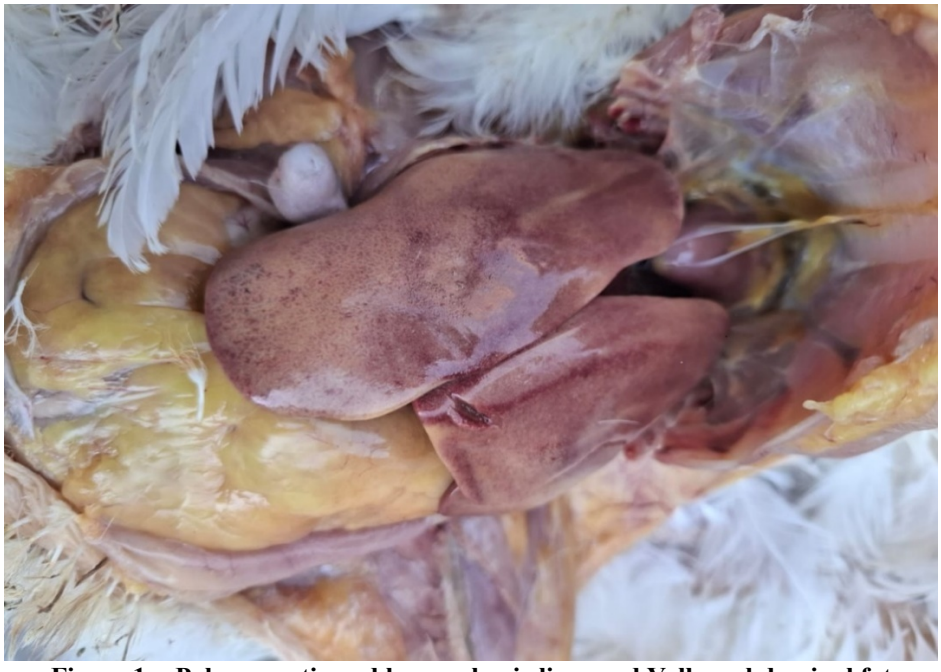


Figure 1a: Pale, necrotic and hemorrhagic liver and Yellow abdominal fat

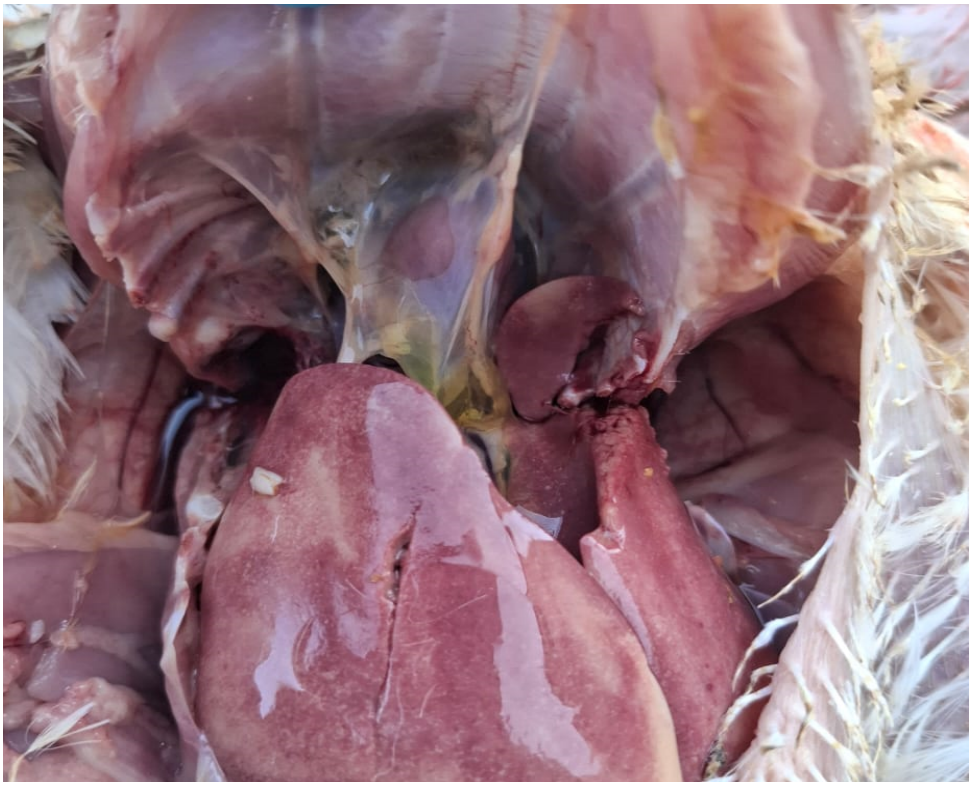


Figure 1b: hemorrhages in liver and fluid accumulation in pericardial sac

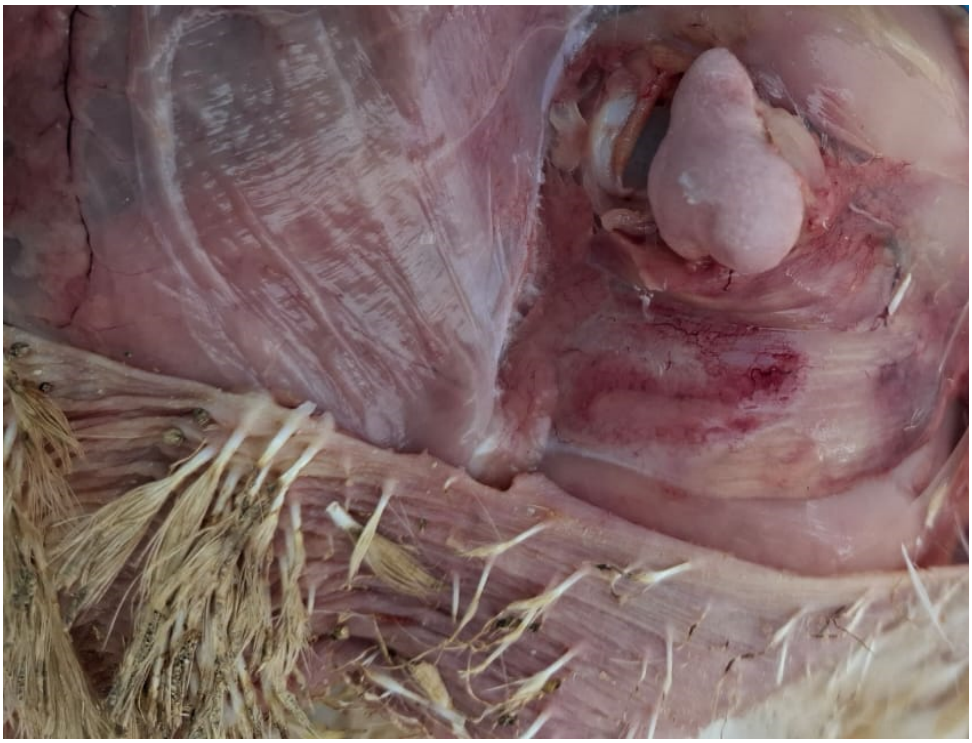


Figure 1c: Heart deformity including expanded atria and constricted ventricles

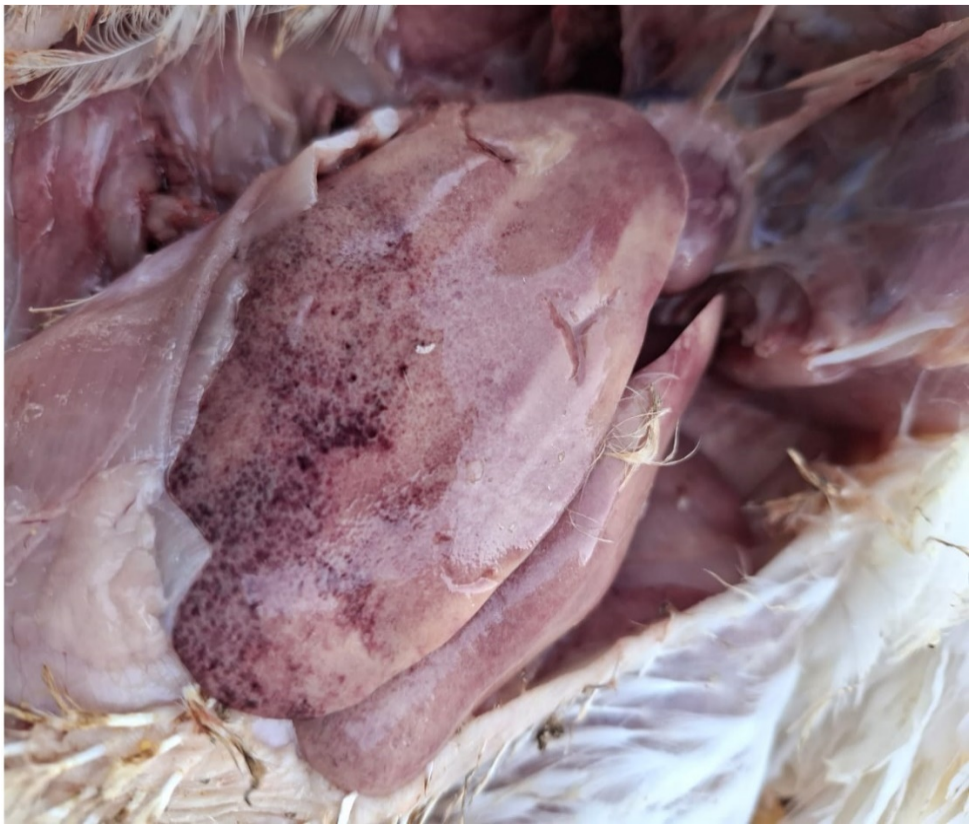


Figure 1d: Necrosis and hemorrhages in liver

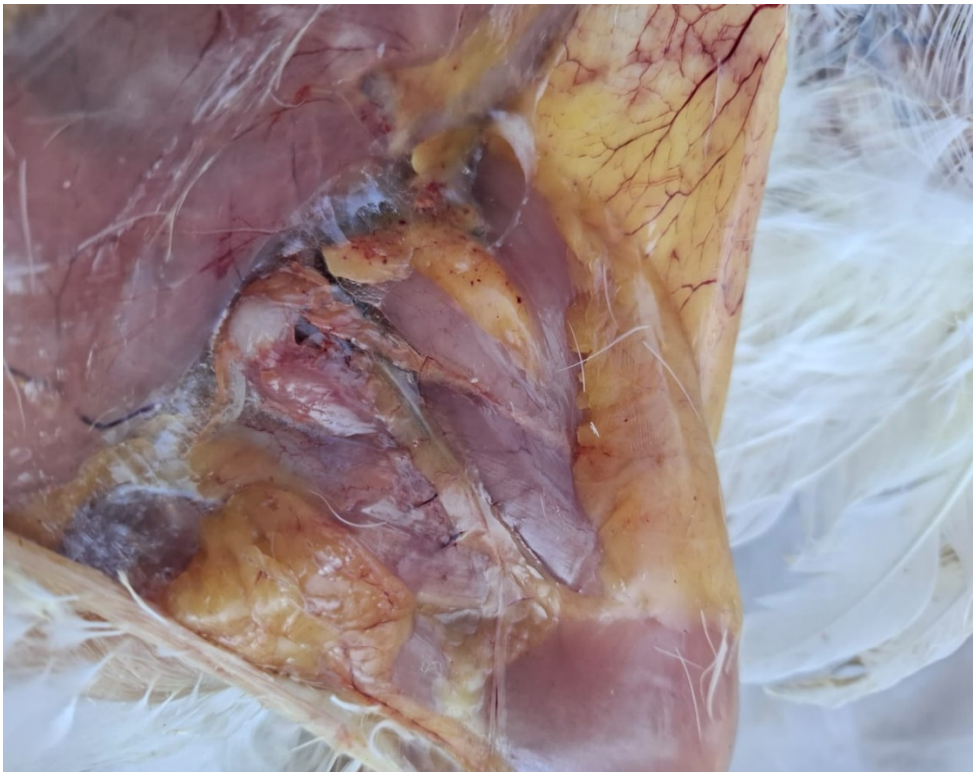


Figure 1e: Yellow abdominal fat and yellow dermis of skin with congested blood vessels and hemorrhages in the muscles

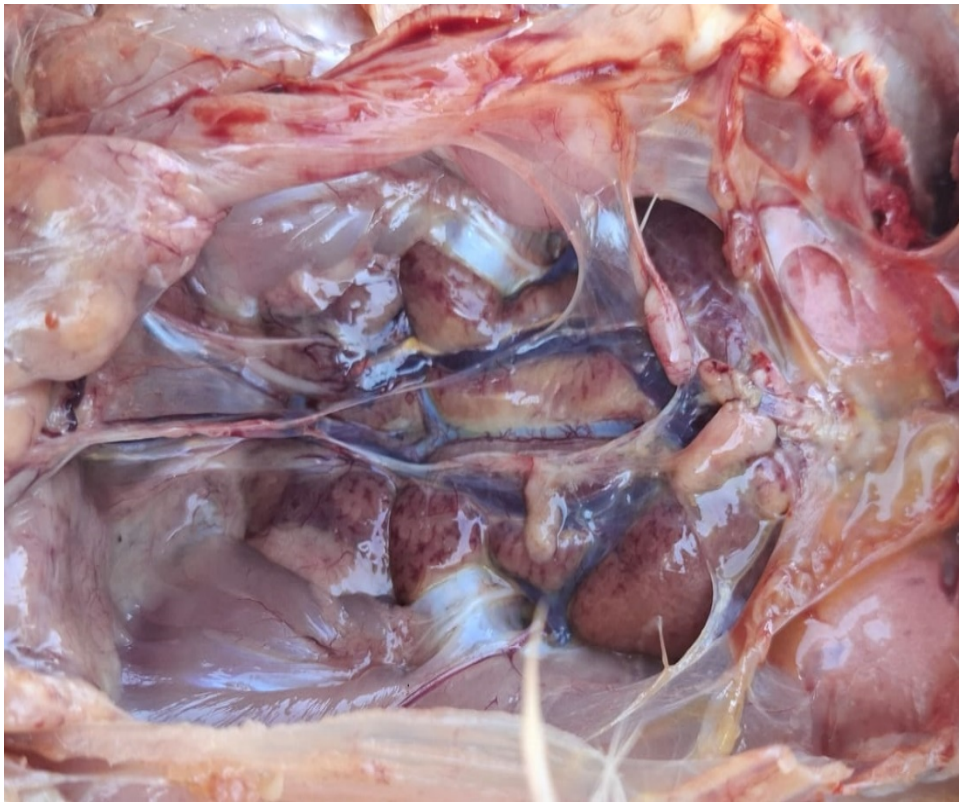


Figure 1f: Congested abdominal blood vessels and hemorrhagic kidney

Figure 1g: Green diarrhea seen in some birds

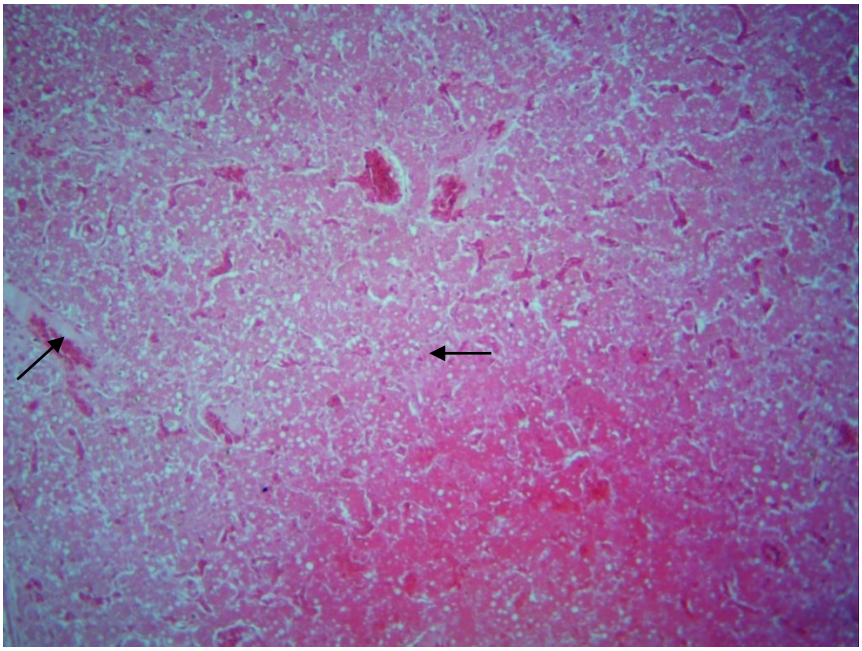


Figure 2a: Congestion and hemorrhage of liver, vacuolation (fatty change) and inclusion bodies in hepatocytes (arrows). H & E stain (100x)

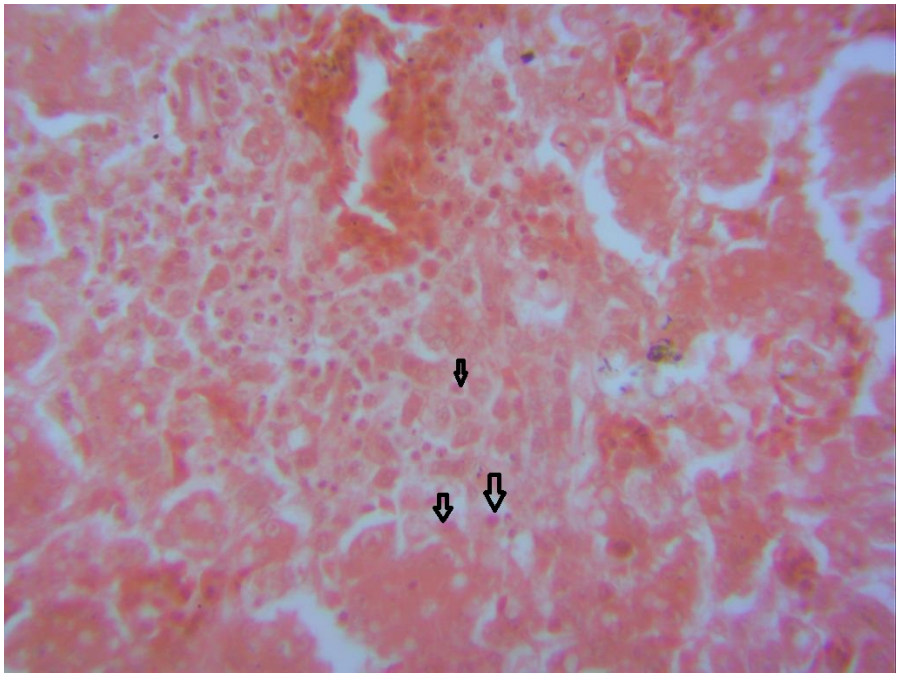


Figure 2b: Inclusion bodies in hepatocytes. Inflammatory cells accumulation H & E stain (400x)

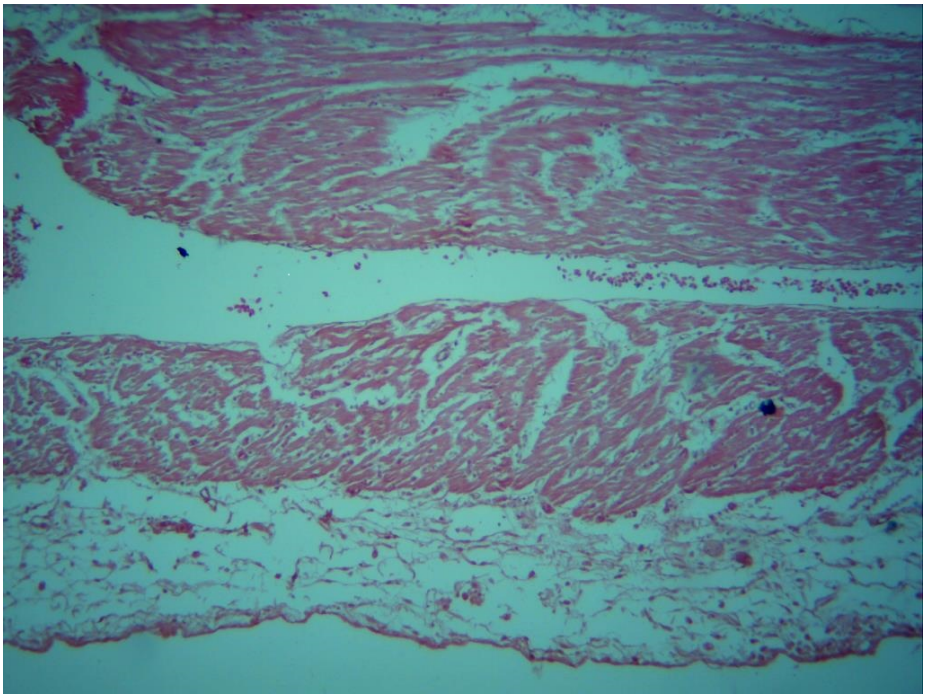


Figure 2c: Expansion of fluid-filled pericardial sac characterized by fibrous and cellular content and myocardiomyocytes necrosis. H & E stain (100x)

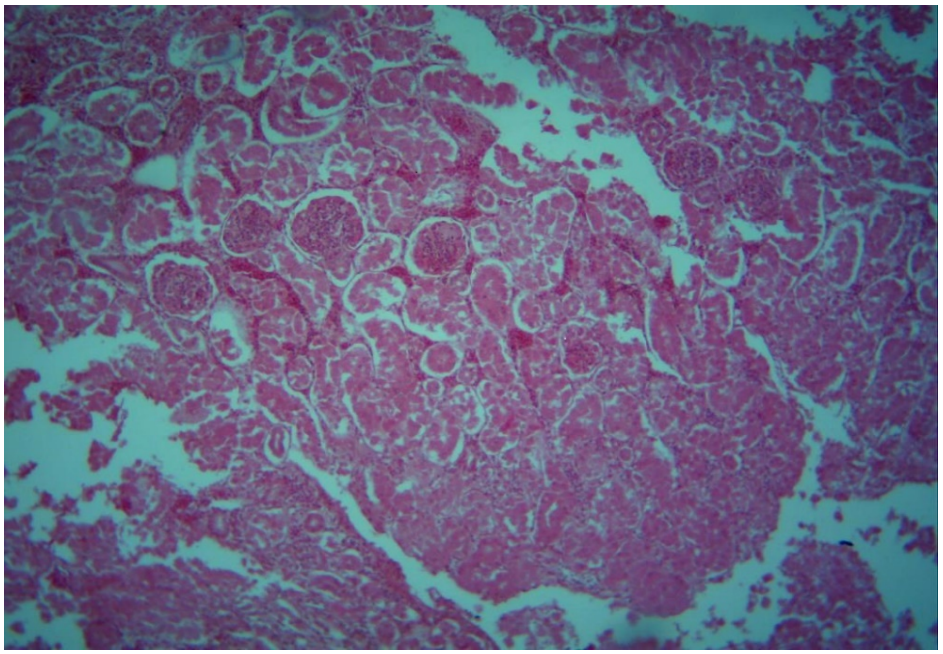


Figure 2d: Renal tubular destruction and necrosis of chicken kidney naturally infected with adenovirus serotype 8b. H & E stain (100x)

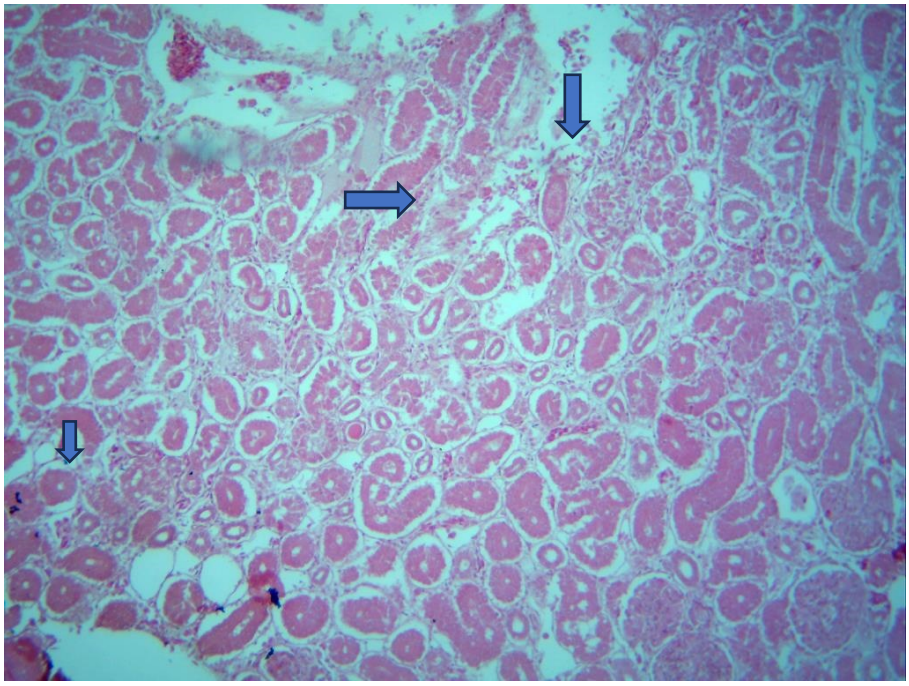


Figure 2e:Renal tubular necrosis (upper arrows) and separation of tubules from its basement membrane (short lower arrow). H & E stain (100x) *

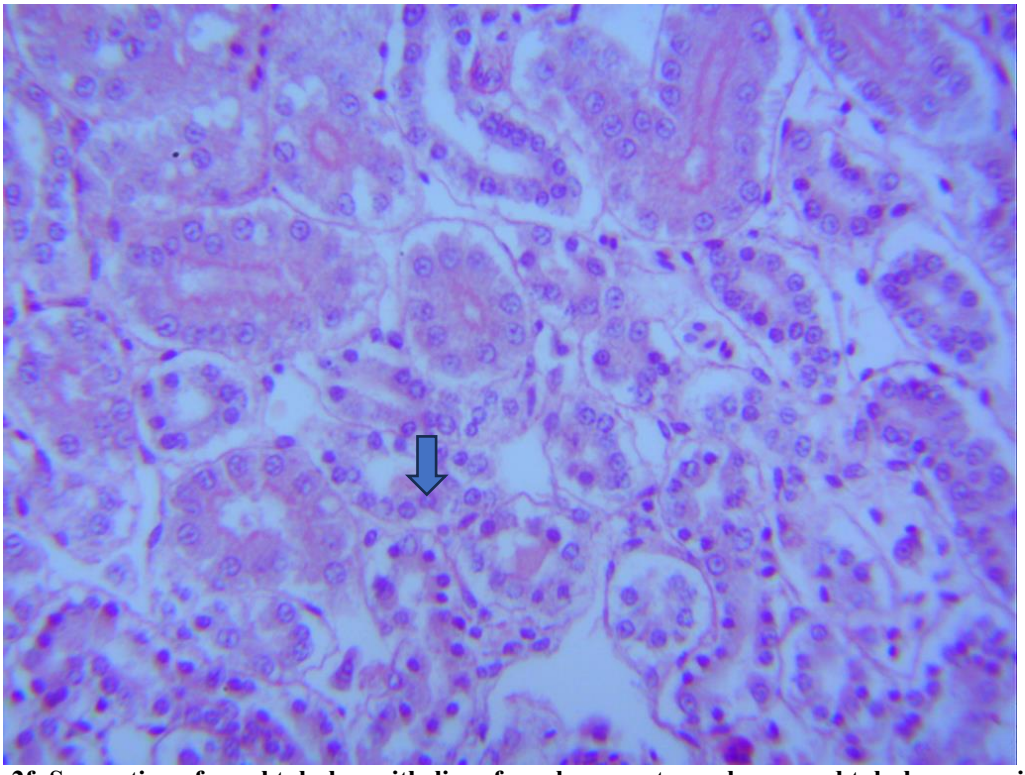


Figure 2f: Separation of renal tubules epithelium from basement membrane and tubular necrosis, and irregular basement membrane (arrow) was noticed (40x). PAS stain

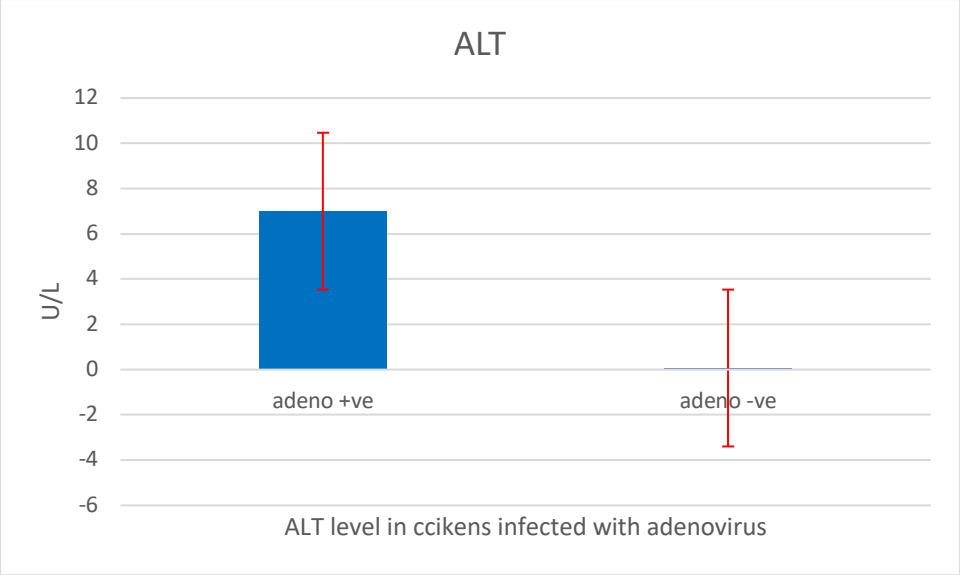


Figure 3a: ALT level in chickens livers infected with adenovirus and non-infected chickens

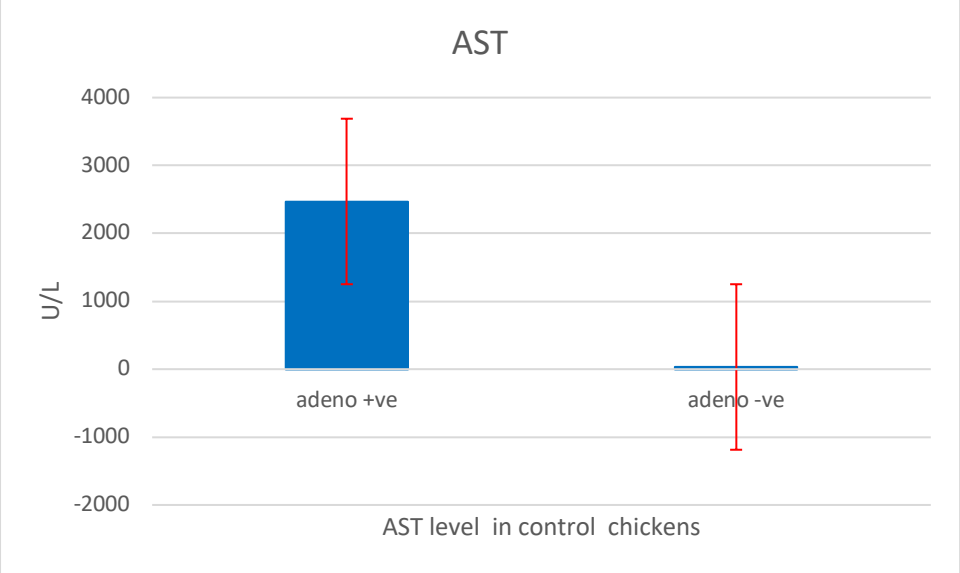


Figure 3b: AST level in chickens livers infected with adenovirus and non-infected chickens

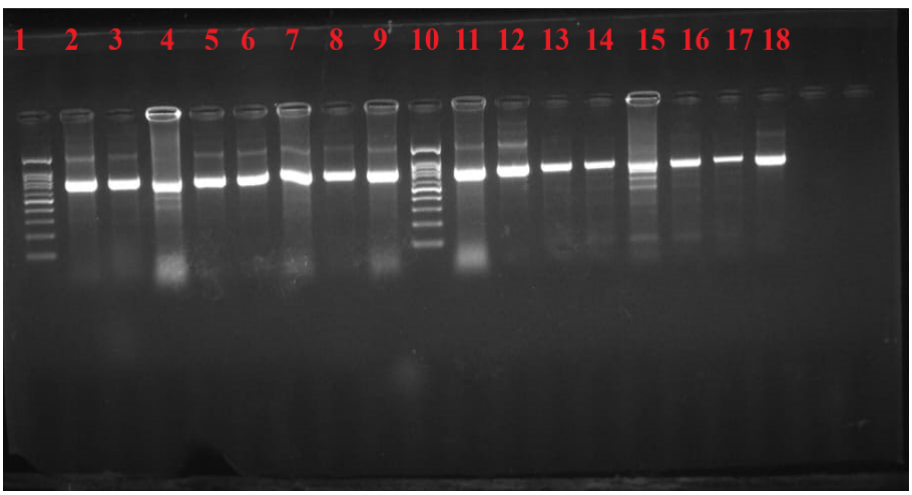


Figure 4a: Amplification of hexon gene of fowl aviadenovirus from examined samples on agarose gel. Lane 1: 100bp DNA ladder. Lane 2-8: liver. Lane 10: 100bp DNA ladder. Lane 11-12: liver. Lane 13-16 heart. lane 17 kidney. Lane 18 blood

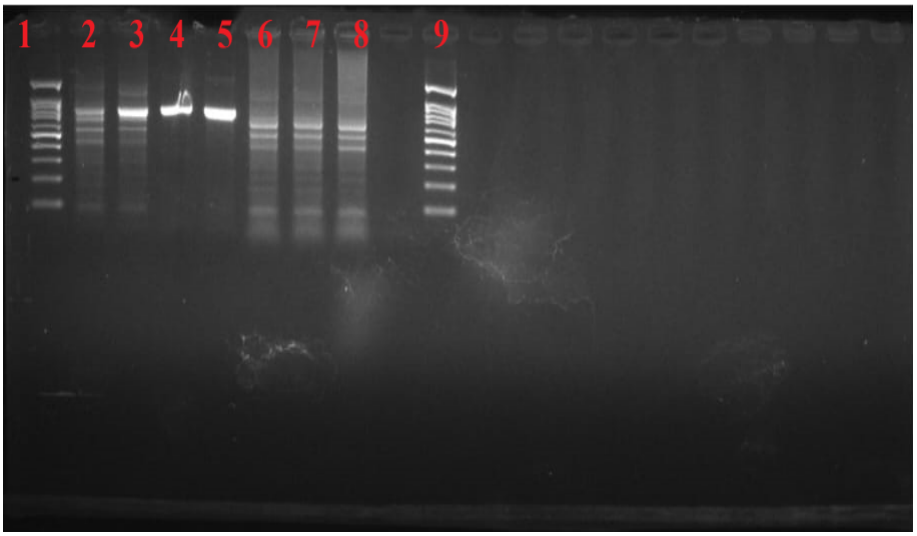
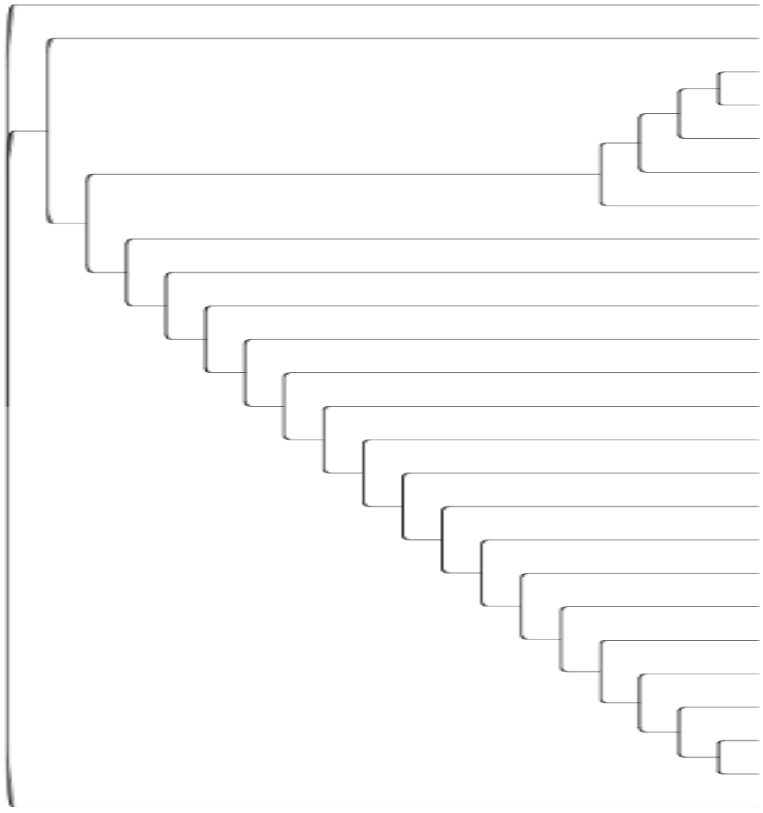


Figure 4b. Hexon gene DNAs of fowl aviadenovirus detected from examined samples, Lane 1: 100bp DNA ladder. Lane 2-4: blood. Lane 5: pericardial fluid. Lane 6-8: negative control. Lane 9: 100bp DNA ladder



MZ368700.1 Fowl aviadenovirus E isolate ISR/4346/2021 hexon gene, partial cds
MK937073.1 Fowl aviadenovirus E isolate TR/BVKE/CA/CAK hexon gene, partial cds
***MN052902.1 Fowl aviadenovirus E strain FAdV-E hexon gene, partial cds**
***MN052902.1 Fowl aviadenovirus E strain FAdV-E hexon gene, partial cds**
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MK937074.1 Fowl aviadenovirus E isolate TR/BVKE/CA/CYG hexon gene, partial cds

Figure 5: Phylogenetic tree of FAdV isolated from positive chicken samples in Jordan. Five isolates in the present study were marked with * and reference strains available at GenBank (the GenBank accession number is shown before the strain)

Discussion

Frequency of inclusion bodies hepatitis in chickens has been rising across the globe causing major loses to poultry industry [26, 27]. It has been established in the earlier research that fowl adenoviruses can be identified in both sub-clinically (apparently healthy) and clinically affected chickens [28,29,30]. Nevertheless, by means of PCR, we failed to isolate the virus from healthy poultry birds in the present study. This can be attributed to the failure to detect viral particles in the livers analyzed from such birds. The presence of viral particles in healthy chickens is also given by the low concentration of ALT and AST. From the data, it was found that adenovirus infected chickens had 100 folds increased levels of ALT and 317 folds increased levels of AST than non-infected chickens. These higher values are clearly suggestive of viral hepatotoxicity in affected chickens and there was no toxic changes in the livers of the non-infected birds, as evidenced by histology. The earlier reports pointing to the isolation of the virus from apparently healthy chickens can be explained by particular circumstances under which birds were as yet in the incubation period, or latent infections. The same viruses could be potential latent infections that could be reactivate under stress conditions like cross species or confinement [30,31,32].

Co-infection of FAdVs with other pathogens are quite common and reported regularly [33,37]. However, our study did not find any co-infections with any immunosuppression diseases associated with adenovirus and this goes in line with the finding of studies who founded that FAdVs are able to suppress the immune system of chickens without prior infection with immunosuppressive agents. This reiterates that co-infection or presence of other viruses may not be critical or necessary for clinical disease conditions as reported earlier by other workers. In our study we isolated adenovirus from blood, hydropericardium, liver, heart, and kidney by PCR. The isolation of the virus simultaneously from these organs and blood indicates ongoing viremia which may persist for more than 5 days. Previous studies have reported that FAdvs are present in all excretions. Adenovirus was successfully isolated from fluids in the pericardial sac by PCR [34,35]. May be, as we see in this current research, the virus can be isolated from any infected organ which show pathological changes and from blood. Theoretically, the virus can be isolated from any organ because of the extended viremia which lasted more than 5 days. Future studies should investigate the duration of viremia to implement more efficient control methods.

This is because the necropsy findings noted in the present study conform to the established findings. There was also hepatitis as shown by the presence of viral inclusion bodies within hepatocytes. Infected birds had pale, enlarged, hemorrhagic, necrotic and friable liver and inflamed and hemorrhagic kidneys. Also, haemorrhage were found in the breast and thigh muscle of the chickens treated with dose [35,36,38]. Deformity in the heart and accumulation of fluids in the pericardial sac and profound jaundice as indicated by the yellow coloration of the abdominal fat and the dermis of the skin.

IBH and HHS share a similar clinical picture, however, HHs is distinguished by the accumulation of straw-coloured fluid in the pericardial sac. Based on this, in addition to PCR and genetic analysis we diagnosed HHS in our study. HHS is a severe syndrome that is characterized by accumulation of clear, yellow or gelatinous fluids in the pericardial sac along with cardiac deformities. The lesions in the liver and the heart are characteristic to HHS [6, 29]. Different lesions are also found in several other organs. liver lesions include multifocal necrosis, intranuclear inclusion bodies in hepatocytes and high mortalities [14]. This form of the disease is caused by serotype 4 of group C [9,30,31]. We for the first time, and up to the best

of our knowledge link group E to HHS as discussed below. The histological findings in our study showed renal tubular necrosis, tubular separation from the underlining basement membrane, and irregular basement membrane structure. Expansion of pericardial space filled with fibrinous and cellular contents and cardiomyocytes necrosis were observed. Liver findings included congestion, hemorrhage, distinctive fatty changes and the presence of typical inclusion bodies in hepatocytes compatible with the diagnosis of inclusion body hepatitis (Figure 2) [22,32,33]. DNA sequencing of the conserved loop 1 of hexon gene is conventional technique used for identification and differentiation of adenoviruses [34]. In this study all the organ and tissue samples subjected to PCR were positive for hexon gene. Molecular identification of the causative agent also determined group E serotype 8b as the responsible strain of HHS. Moreover, this is the first comprehensive study in which a FAdV Jordanian isolate is described and a relationship between group E FAdV and HHS in broiler poultry is evidenced. At the present time, no vaccination against adenoviruses is carried out in Jordan because nothing is known about the prevailing serotypes of the virus in the region, and this disease has not been described in the country before. This is because there are no or limited cross reactions between the strains which make the use of vaccines containing multiple serotypes to be more effective. While an inactivated FAdV-4 vaccine is quite effective and has already provided experimental cross protection against serotypes 8b and 11 [31], they noted no field cross protection [35]. However, this development demands more wanton molecular epidemiological studies in Jordan as well as in other parts of the globe in order to determine the current status of FAdV. These studies will aid in the future formulation and execution of appropriate controlling means namely shots in specific subgroups. Moreover, the identification of a new factor in HHS that can be caused by group E serotype 8b, besides its known contribution to IBH, has important implications for disease prevention. Further studies are required to find out whether all serotypes of the group E and other groups can cause HHS with the known ability of IBH.

Conclusion

Therefore, this study proves the importance of studying FAdV infections in poultry with particular emphasis on the FAdV-8b serotype as the cause of HHS in Broiler chickens. Pathological tests such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) depicted severe liver lesion in the infected birds as compared to being healthy uninfected chickens. PCR work confirmed the identification of the hexon gene in tissues from different body parts of infected birds, establishing adenovirus as the causative agent. This is the first attempt made at isolating, identifying and characterizing FAdV in Jordan, the association of FAdV group E with HHS, and the playing out of ascertainable economic losses in the poultry industry. Lack of vaccination campaigns in Jordan makes molecular epidemiology population studies important in identifying circulating serotypes and the appropriate type of vaccine to develop. The results of this work will enhance knowledge of the pathogen FAdV and demonstrate the necessity for diversified control measures to limit its negative effects on poultry production.

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- Conceptualization, Omran H. Alameri, Mu'ath Q. Al-Ghadi, Anas Al Makhzoomi and Juhina Salim Ababneh
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- Formal analysis, Omran H. Alameri and Juhina Salim Ababneh
- Investigation, Omran H. Alameri; resources, Mu'ath Q. Al-Ghadi and Omran H. Alameri Data curation, Omran H. Alameri and Mu'ath Q. Al-Ghadi
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- Visualization, Anas Al Makhzoomi, Omran H. Alameri, Mu'ath Q. Al-Ghadi
- Supervision, All authors; project administration, Mu'ath Q. Al-Ghadi; funding acquisition, Mu'ath Q. Al-Ghadi.
- All authors have read and agreed to the published version of the manuscript."

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