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RESEARCH ARTICLE

Outbreaks of Inclusion Body Hepatitis Caused by Fowl Adenovirus in Commercial Broiler Farms in the Kurdistan Region, North Iraq from 2013 to 2021

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A B S T R A C T

Inclusion body hepatitis (IBH) is a disease caused by fowl adenovirus (FAdV), categorized under the Adenoviridae family. FAdVs are distributed globally but have not been documented in Iraq since 1979. This study reports the disease occurrence in 89 broiler farms in Sulaymaniyah, Kurdistan Region, North Iraq, from April 2013 to April 2021. Infected birds' ages ranged between two days and four weeks. Clinically, birds were lethargic, huddling with ruffled feathers, lacked appetite, and showed yellowish mucoid droppings. The gross lesions included enlarged mottled liver, pale to icteric skin, swollen and pale kidneys, and hemorrhage on the skeletal muscle. Also, histopathological examinations revealed large intranuclear inclusion bodies in hepatocytes, degeneration and congestion of liver sinusoids, degeneration of renal tubules, interstitial tissue infiltrated with inflammatory cells, and necrotizing pancreatitis. PCR was used to detect the virus by amplification of partial 1300 bp *hexon* gene. The amplified fragments were confirmed by sequencing. The study results indicate that avian adenovirus is enzootic in Sulaymaniyah, Kurdistan Region of Iraq. Two different subgroups circulate in Kurdistan. The FAdV/Kurdistan/2013, FAdV/Kurdistan/2020, and FAdV/Kurdistan/2021 belong to the FAdV-E subgroup. On the other hand, the FAdV/Kurdistan/2015 belongs to the FAdV-D subgroup.

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INTRODUCTION

Adenoviruses (AdVs) are unenveloped icosahedral viruses belonging to the Adenoviridae family. The family contains five genera: Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, and Siadenovirus (Zhao *et al.*, 2016). AdVs have been isolated from various animals (Pénzes *et al.*, 2014). Chickens affected by Aviadenovirus develop inclusion body hepatitis (IBH) (Zhao *et al.*, 2016). Fowl adenoviruses (FAdVs) induce many diseases in chickens like adenoviral gizzard erosion (AGE), hydropericardium hepatitis syndrome (HHS), and IBH, leading to economic losses everywhere in the world (Abdul-Aziz and Hasan, 1995; Swar and Shnawa, 2021).

FAdV-D and FAdV-E are the main FAdV species isolated from IBH cases (Morshed *et al.*, 2017; Cizmecigil *et al.*, 2020). FAdV-4 (FAdV-C) strains are responsible for causing HHS and are extremely infective to chickens (Hess, 2000). FAdV-1 (FAdV-A) has been recovered from gizzard erosion cases (Ono *et al.*, 2003).

The first IBH report was from the USA in 1963 as necrotizing hepatitis in seven-week-old chickens (Mettifogo *et al.*, 2014). Later, the disease was reported in several areas. In 1988, a new disease, called Angara Disease, was reported in broilers from Angara Goth near Karachi in Pakistan (Shah *et al.*, 2017). The disease course and clinical signs were almost similar to IBH. The pathological findings included clear, straw-colored fluid accumulation in the pericardial sac known as

hydropericardium syndrome (El-Tholoth and Abou El-Azm, 2019). The disease has also been recorded in Iraq in 1979 (Hess, 2017), but other outbreaks have not been reported since then.

IBH is transmitted vertically and horizontally, but the former is reported as a very effective means of spreading from parent birds to off-springs (Gomis *et al.*, 2006). Horizontal infection occurs through the fecal-oral route and spreads mechanically (Hafez, 2011).

Over the past 20 years, increasing IBH outbreaks have been reported in several areas, stressing the disease's global occurrence (Schachner *et al.*, 2018; Wang *et al.*, 2020). IBH mainly affects broilers aged up to 35 days, but the disease has also been described periodically in all age groups. The disease was reported in birds as young as seven day-olds and as old as five months. In natural outbreaks, IBH is characterized by 2–40% mortalities in chickens, and high death rates occur in birds younger than 21 days. Depending on the virus's pathogenicity, chicks' immune status, and simultaneous secondary infections, mortality as high as 80% may occur. Generally, mortality peaks within 3-4 days and falls in 9–14 days (Ahamad *et al.*, 2016).

In most cases, the liver is the primarily affected organ. Grossly, IBH lesions include hepatomegaly with a pale, friable liver and occasional necrotic foci. Ecchymotic hemorrhages might also be observed in the leg, breast muscles, and liver. Clinical signs are not conclusive, including lethargy, huddling, ruffled feathers, and appetite loss (Asthana *et al.*, 2013; Wang *et al*., 2020).

The definitive diagnosis of IBH is primarily based on polymerase chain reaction, histopathological examinations, or virus or antigen detection using immunofluorescence test or electron microscopy (Mittal *et al.*, 2014; Hosseini *et al.*, 2020). Histopathological lesions include focal necrotic areas and intranuclear inclusion bodies in some hepatocytes, and the inclusion bodies might be eosinophilic or occasionally basophilic. They occur as large, round, or irregularly shaped structures with a clear pale halo (Hemida and Alhammadi, 2017).

IBH or similar cases characterized by hepatitis and hepatocytic intranuclear inclusion bodies have not been documented in poultry in the Kurdistan region of Iraq. Therefore, the cases of adenovirus-like IBH in broiler farms in Kurdistan of Iraq are reported here for the first time.

MATERIALS AND METHODS

Field samples and clinical findings: The study was administered from April 2013 to April 2021, covering about 1300 broiler farms from different Sulaymaniyah governorate areas, Kurdistan/Iraq. The research was conducted following the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. The protocol was approved by The Animal Care and Use Committee (ACUC) at the College of Veterinary Medicine/University of Sulaimani (Approval No. 20/6). Also, permission for participation was obtained from each farm owner upon collecting the tissue samples.

Outbreaks occurred in 89 farms between April 2013 and April 2021. The birds showed a sudden onset of high mortality, lethargy, ruffled feathers, and inappetence. The livers were the primarily affected organs at necropsy, which were enlarged, pale yellow with necrotic foci and multiple petechiae, enlargement of kidney, and hemorrhage in skeletal muscles. Four chickens with suspected clinical signs were selected from each farm, and samples were collected from the liver, kidneys, and pancreas. After that, the samples were transported with ice bags in a coolbox to the Molecular Diagnostic Laboratory for direct DNA extraction. For histopathology examination, tissue samples of the liver, kidneys, and pancreas were fixed in a 10% neutral-buffered formalin (NBF) solution.

Histopathological examination: Tissue specimens preserved in 10% NBF were processed using a routine paraffin embedding procedure. Five micrometer-thick sections were stained with routine hematoxylin and eosin and studied under a light microscope for histological changes associated with FAdV infection (Suvarna *et al.*, 2018).

DNA extraction: Total DNA was extracted from 20–35 mg of pooled tissue specimens of liver, kidney, and spleen following the Genomic DNA Extraction Kit (AccuPrep® by Bioneer, Korea) manufacturer's instructions.

PCR amplification: The PCR reaction was accomplished in 200 µL tubes using AccuPower PCR PreMix. The tube content comprised 5 µL DNA, 1 µL (10 pmol) H3 forward primer AACGTCAACCCCTTCAACTACC, and 1 µL (10 pmol) H4 reverse primer TTGCCTGTGGCGAAAGGCG to amplify the 1300 bp of the partial hexon gene (Raue and Hess, 1998). The volume was then completed to 20 µL using DEPC-H_2O. The thermocycler was programmed to start with denaturation at 94°C for 5 minutes. Then, 35 denaturation rounds at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 90 seconds followed. The last elongation temperature was 72°C for 10 minutes.

Electrophoresis and sequencing: PCR products' electrophoresis was done on a 1% agarose gel. Seven microliters of the PCR product were stained with 3 µL safe green dye and visualized by a UV transilluminator. The sizes of the PCR products were approximated according to a 100 bp DNA ladder migration pattern. The PCR product was sequenced from both terminals by the Macrogen sequencing service in South Korea, and the DNA sequence was published in GenBank as Fowl adenovirus isolate FAdV/Kurdistan/2013 accession number KF601576, FAdV/Kurdistan/2015 accession number KU060147, FAdV/Kurdistan/2020 accession number MN967070, and FAdV/Kurdistan/2021 accession number MZ053469.

Phylogenetic analysis: The blast method determined the partial hexon gene sequence identity of all Kurdistan FAdV isolates at the National Center for Biotechnology Information (NCBI) homepage. A phylogenic tree was constructed by nucleotide sequences of the *hexon* gene of 34 FAdV isolates of different genotypes according to a neighbor-joining technique using the Kimura2-parameter model Mega 10. The bootstrap numbers were decided from 1000 replicates of the original data (Tamura *et al.*, 2011).

RESULTS

Clinical manifestation and postmortem findings: The current study focused on infected chickens of the Ross breed. IBH occurred in 89 farms around Sulaymaniyah city in the Kurdistan of Iraq, and the clinical signs in 2-3-week-old broiler chicks included lethargy, huddling with ruffled feathers, inappetence, and greenish diarrhea.

The mortality rates ranged between 8% and 15%. Postmortem findings included pale icteric skin and ecchymotic hemorrhages on the skeletal muscles (Fig. 1A). Enlarged, pale, and mottled liver (Fig. 1B and 1C), swollen pale kidneys with distended tubules (Fig. 1D), and pale and swollen pancreases were detected. Also, the bursa of Fabricius and thymus had not atrophied, and the bone marrow was red.

Histopathology: The most critical histopathological finding in liver sections of diseased birds was the presence of inclusion bodies in the hepatocytes (Fig. 2), appearing as large basophilic intranuclear inclusion bodies with a pale boundary. Many hepatocytes were degenerated and swollen with vacuolated cytoplasm. Infrequently, necrosis of hepatocytes had occurred. Moreover, there was lymphocytic and heterophilic hepatitis. These inflammatory infiltrates appeared clearly around the central vein (Fig. 2B).

Additionally, there was dilation and infiltration of sinusoids with lymphocytes and heterophils. Histopathological changes also involved the kidneys and pancreas comprising degeneration and necrosis of the renal tubule with intranuclear inclusion bodies and infiltration of the mononuclear inflammatory cells within interstitial tissues (Fig. 3). The pancreas was affected with large areas of necrosis, complete loss of pancreatic acinar architecture, hemorrhage, and basophilic intranuclear inclusion bodies (Fig. 4).

Detection of FAdV isolates: Fowl adenovirus was detected using partial *hexon* genes of FAdV. The expected amplicon size was 1300 bp of FAdV, detected in four pooled samples from different broiler farms. The amplified fragments were confirmed by sequencing.

Phylogenetic analysis of FAdV isolates: A phylogenetic tree was constructed based on the partial hexon gene of FAdV sequence alignment of the 34 isolates (Fig. 5). The FAdV isolates were divided into five general clusters: FAdV-A, FAdV-B, FAdV-C, FAdV-D, and FAdV-E. The tree's topology indicated two different FAdV genotypes with different genomic evolutions. The FAdV/ Kurdistan/ 2013, FAdV/Kurdistan/2020, and FAdV/ Kurdistan/2021 belonged to the FAdV-E genotype. On the other hand, the FAdV/Kurdistan/2015 belonged to the FAdV-D genotype and closer to the Saudi Arabia virus isolated in 2015 (MK995481).

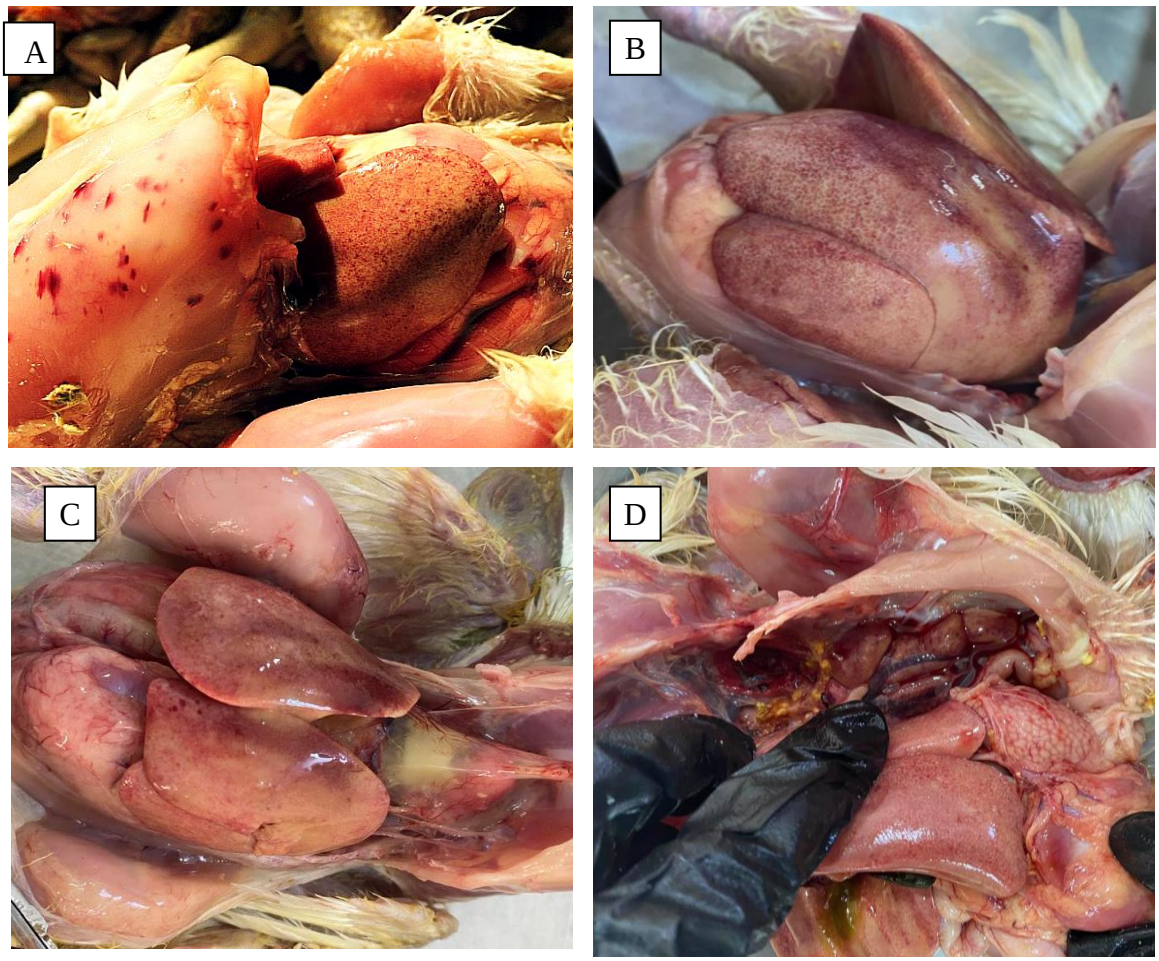

Fig. 1: Gross lesions in infected broilers with fowl adenovirus. A: Ecchymotic hemorrhages in the skeletal muscles, B: Hepatomegaly, C: Friable liver with petechial hemorrhage, D: A clinical case in which there is an enlargement of the liver with small white necrotic foci and petechial hemorrhagic spots.

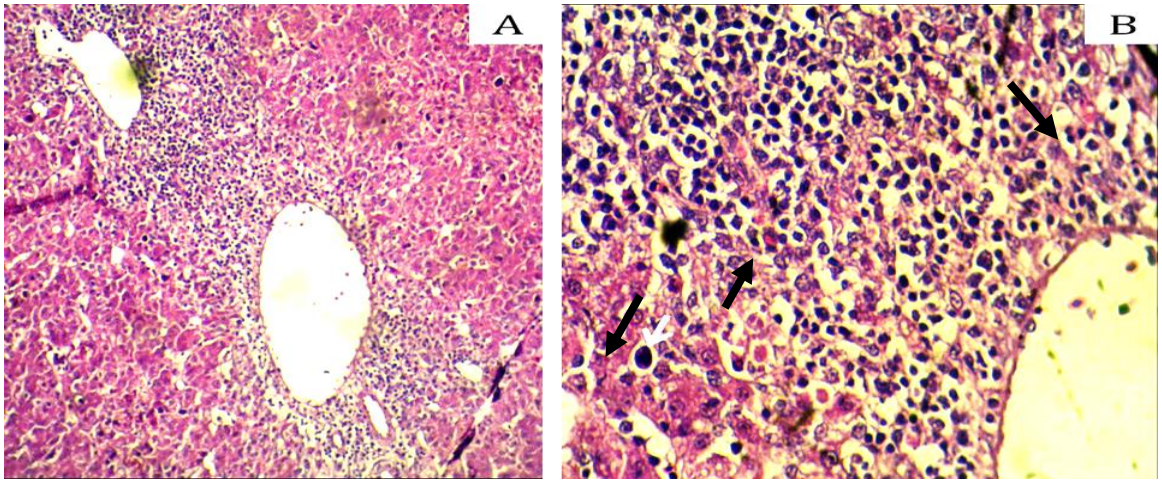

Fig. 2: Histopathological sections of the liver. (A) There are moderate lymphocytic and heterophilic inflammatory infiltrates surrounding the central vein. Diffuse cytoplasmic vacuolation is observed within remaining hepatocytes, and there is dilation and infiltration of sinusoids with lymphocytes, heterophils, and histiocytes. (B) In the previous slide, with higher magnification, the nuclei of numerous hepatocytes contained one large basophilic inclusion body (arrows), a halo is present around the intranuclear inclusion, and the nucleus membrane was hyperchromatic (H and E stain, X100, X400).

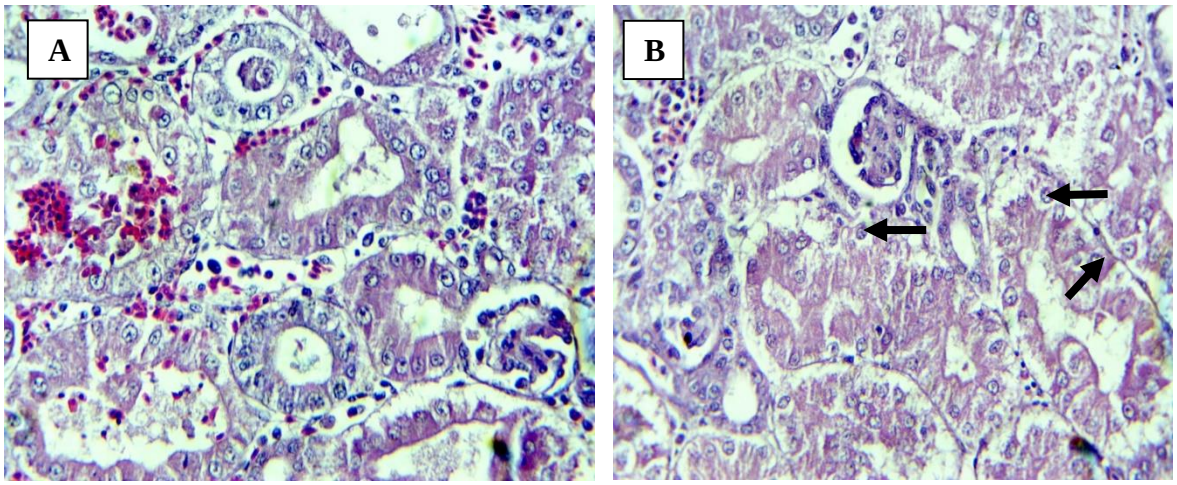

Fig. 3: Microscopic sections of the kidneys showing A: degeneration and necrosis of renal tubular epithelium, B: focal hemorrhages and interstitial mononuclear cell infiltration with intranuclear inclusion bodies (Arrows) (H and E stain X400).

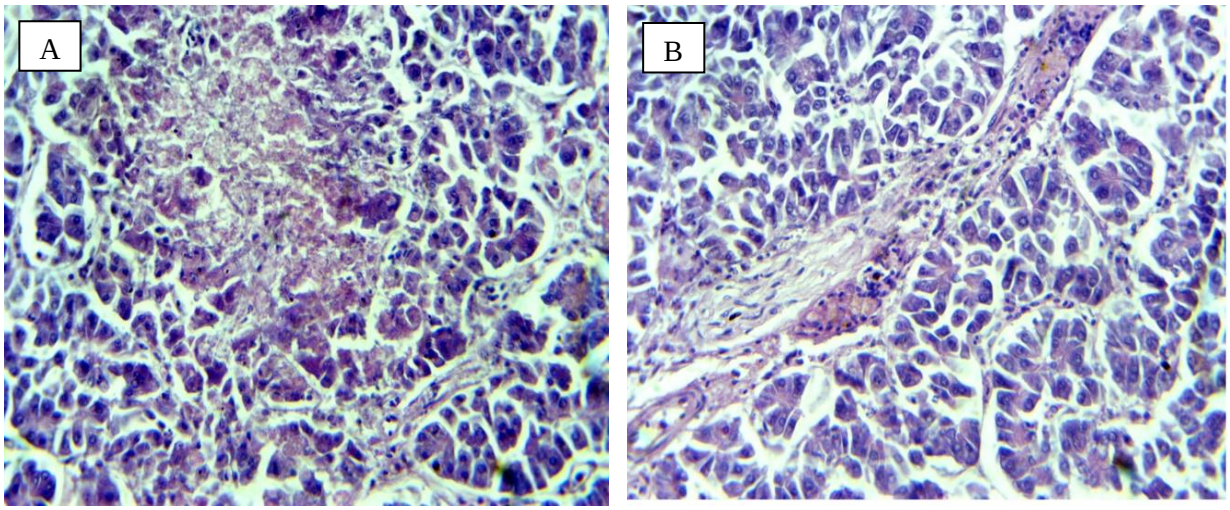

Fig. 4: Microscopic sections of the pancreas. A: Large areas of necrosis characterized by loss of pancreatic tissue and replacement by cytoplasmic and nuclear debris with B: Some heterophilic infiltration involving acinar tissue. Several acinar cells around the lesion contain large, deeply basophilic, intranuclear inclusion bodies

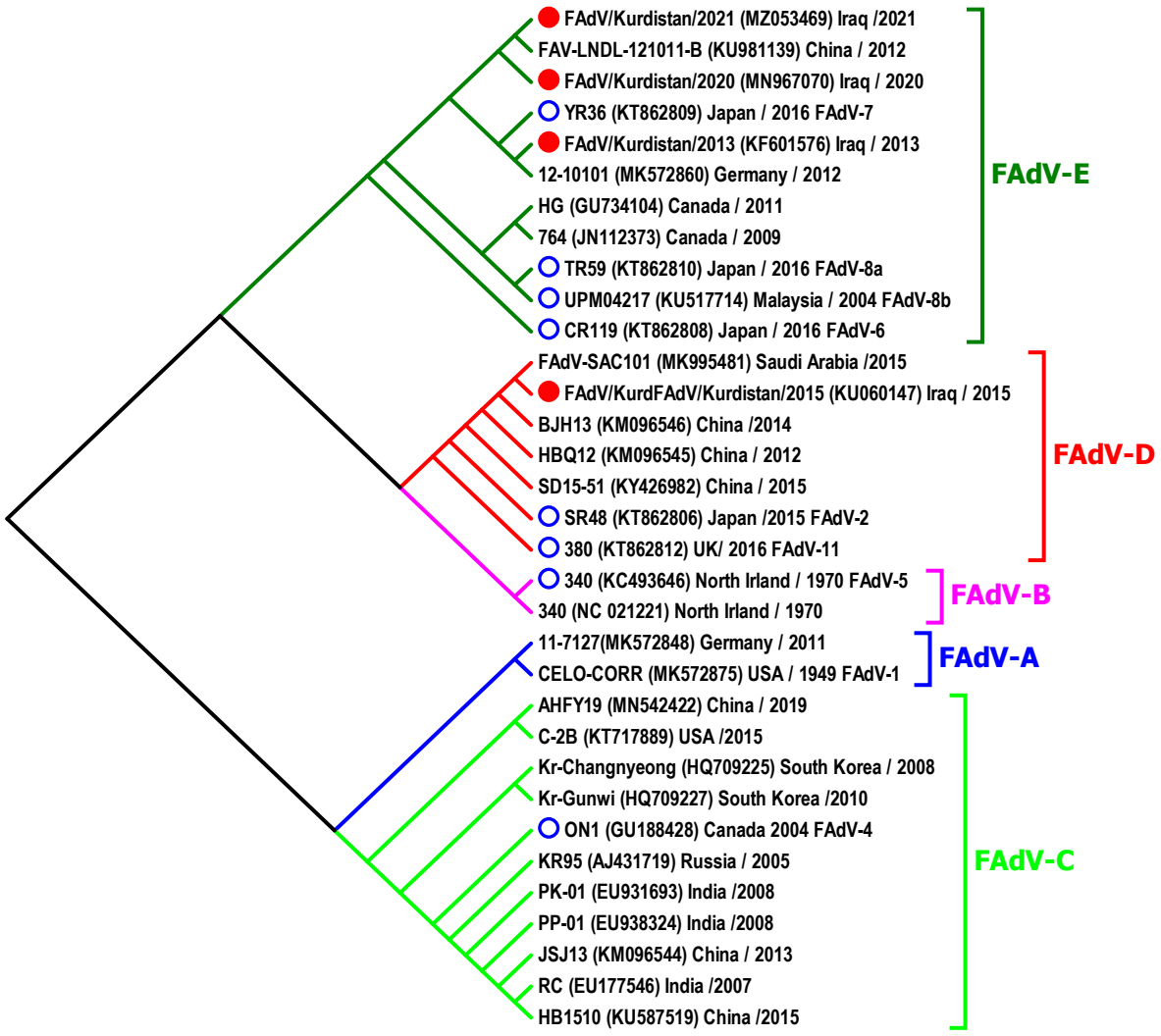

Fig. 5: Phylogenetic tree of Kurdistan FAdV isolates. The phylogenetic tree analysis according to partial *hexon* nucleotide sequence indicates five clusters. The red circle indicates detected isolate in this study, and the blue circle indicates the reference genotypes. A phylogenic tree was based on the neighbor-joining method using the Kimura2-parameter model Mega 10. The bootstrap values were determined from 1000 replicates.

DISCUSSION

Iraqi Kurdistan contains more than 1300 poultry farms, and intensive farming and deficient control strategies have facilitated different viral infections. Thus, epidemiologic studies are crucial to monitor disease outbreaks and vaccine development. FAdV outbreaks have become a big problem facing poultry farming globally (Pan *et al.*, 2017). IBH causes mortality resulting in production and economic losses. More FAdV clinical cases have been reported recently, and several FAdV strains have been isolated from diseased birds in many areas (Rahimi and Haghighi, 2015; Pan *et al.*, 2017). There is no published data about FAdV in Kurdistan broiler farms, reinforcing the requirement for molecular surveys on this emerging infectious agent in poultry and studying its fundamental role in clinical diseases. This study describes IBH outbreaks in Kurdistan/Iraq broiler flocks for the first time.

Mortality from IBH outbreaks is usually 2–10%, but it can reach 30% in cases of coinfection with other immunosuppressive cases. In this study, sudden high mortality of 8% to 15% started at two days to four weeks of the infected broilers' age, consistent with previously published literature (Alvarado *et al.*, 2007). Although IBH can infect chickens of all ages; however, in this study, young chicks were more sensitive during the first 14 days. This outcome indicates that there is an apparent age effect with avian adenoviruses. As the host's age increases, the multiplication of the viruses within the host becomes confined, and the mortality reduces (Cizmecigil *et al.*, 2020).

Distinctive necropsy findings such as hepatomegaly, enlarged and hemorrhagic spleen and kidneys, and clinical findings were detected in this study's IBH cases. Also, other scientists described the enlarged pale pancreas and greenish diarrhea in some chicks (Schachner *et al.*, 2018).

The most critical histopathological finding in liver sections of diseased birds is the presence of large basophilic intranuclear inclusion bodies in hepatocytes surrounded by a pale halo, also previously reported (Rahimi and Haghighi, 2015). Many hepatocytes were degenerated and appeared swollen with vacuolated cytoplasm. Infrequently, there was necrosis of hepatocytes and lymphocytic and heterophilic hepatitis. These inflammatory infiltrates appear clearly around the central vein, which was also reported by Oliver-Ferrando *et al.* (2017). Additionally, there was dilation and infiltration of sinusoids with lymphocytes and heterophils.

Based on phylogenetic relationships, pathogenicity, restrictive fragmentation, and cross-neutralization of FadVs, they are divided into five species, A to E (Wajid *et al.*, 2018). In the present study, the isolates' phylogenetic analysis revealed the circulation of two different FAdV species in Kurdistan. Both Kurdistan genotypes had different genomic evolution. The FAdV/Kurdistan/2013, FAdV/Kurdistan/2020, and FAdV/Kurdistan/2021 belong to FAdV-E, agreeing with another study in a Korean epidemiologic FAdV outbreak that showed FAdV-D were relevant to IBH lesions (Niczyporuk, 2017). On the other hand, the FAdV/Kurdistan/2015 belonged to FAdV-D, closer to the isolated virus Saudi Arabia from chicken and falcon in 2015 (Mohamed *et al.*, 2018). This result proposes a possible virus spread from these areas, perhaps *through* migratory birds. The phylogenetic trees and sequence alignments indicated FAdV-E as the prominent epidemic strain in the IBH outbreak in Kurdistan. However, the circulation of two FAdV genotypes in Kurdistan may exacerbate the clinical disease because some strains of different genotypes can simultaneously reproduce IBH and hydropericardium syndrome (Zhao *et al.*, 2015).

Immunosuppressors such as Infectious bursal disease virus, chicken infectious anemia virus, and Marek's disease virus contribute to IBH outbreaks propagation or worsen clinical signs of FAdV infections (Schat and Skinner, 2014). However, several studies showed that IBH might occur as a primary disease (Morshed *et al.*, 2017; Revajova *et al.*, 2017). The outbreaks reported in this study indicated that IBH can occur as a single infection causing different morbidities and mortalities and that better control programs are required to reduce the economic losses caused by the disease in the Kurdistan Region, North Iraq.

Conclusions: This study is the first molecular characterization and histopathological examination of FAdVs in Sulaymaniyah broiler farms. Two FAdV genotypes circulate in the region, showing that FAdV-7 may have spread from a mutual ancestor to become the predominant epidemic genotype in the most recent IBH outbreaks in Sulaymaniyah. FAdVs could be emerging infectious agents in Kurdistan poultry flocks, causing severe disease in young chicks. More research is needed to evaluate fowl adenoviral genotypes' prevalence and pathogenicity in poultry farms in Iraq. Determination of FAdV genotypes is essential in epidemiologic surveys of the disease outbreaks, development of preventative steps, and adoption of vaccination programs. There is also a need for future development in molecular methods to identify adenovirus strains' origins in IBH.

Ethics approval and consent to participate: The research was conducted following the National Institute of Health's Guide for the Care and Use of Laboratory Animals. The study protocol was submitted to and approved by The Animal Care and Use Committee (ACUC) at the College of Veterinary Medicine, University of Sulaimani (Approval No. 20/6).

Availability of data and materials: The DNA sequences were published in GenBank as Fowl adenovirus isolate FAdV/Kurdistan/2013 accession number KF601576, FAdV/Kurdistan/2015 accession number KU060147, FAdV/Kurdistan/2020 accession number MN967070, and FAdV/Kurdistan/2021 accession number MZ053469.

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Authors contribution: Conceptualization, NMS, EAA, RRS, and NRA; Writing, HOD and PMA; Data analysis, NMS, SFM and ZHM; PMA and OID; All authors have read and agreed to the published version of the manuscript.

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