Identification of Fowl Adenovirus as a causative agent of inclusion body hepatitis (IBH) in commercial broilers

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March 07, 2024

Abstract

Inclusion body hepatitis is a viral disease caused by Adenovirus group I, and it is worldwide in distribution. The virus is endemic in Sulaymaniyah city, Kurdistan Region of Iraq, and infections occurred in forty-six broiler farms from April 2013 to May 2020. Infected bird's ages ranged between two days and four weeks. Clinically, birds showed lethargy, huddling with ruffled feathers, inappetence, and yellow, mucoid droppings. Gross lesions included enlarged mottled liver, pale icteric skin, swollen pale kidney, and hemorrhage on the skeletal muscle. Histopathological examinations revealed large intranuclear inclusion bodies in hepatocytes, degeneration and congestion of liver sinusoids, and degeneration of renal tubules, spermatozoa, spermatid, Sertoli cells, and Leydig cells in the testicle. There were intertubular hemorrhages and large vacuoles. The seminiferous tubular lumens were dilated, contained necrotic debris, and were devoid of spermatozoa in the interstitial tissue. Lesions in the testicle are reported for the first time in the present study. RT-PCR was used to detect the virus by amplification of partial 1300 bp hexon genes. The amplified fragments were confirmed by sequencing. Our results concluded that two different genotypes circulate in Kurdistan, and the nucleotide sequence of Kurdistan fowl adenovirus (FAdV) isolates show only 81% homology together. The FAdV/Kurdistan/2013 and FAdV/Kurdistan/2020 belonged to FAdV-E close to USA isolates. On the other hand, the FAdV/Kurdistan/2015 belonged to FAdV-D closer to the Chinese FAdV isolate.

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Short running title: Inclusion body hepatitis by Fowl Adenovirus

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Abstract

Inclusion body hepatitis is a viral disease caused by Adenovirus group I, and it is worldwide in distribution. The virus is endemic in Sulaymaniyah city, Kurdistan Region of Iraq, and infections occurred in forty-six broiler farms from April 2013 to May 2020. Infected bird's ages ranged between two days and four weeks. Clinically, birds showed lethargy, huddling with ruffled feathers, inappetence, and yellow, mucoid droppings. Gross lesions included enlarged mottled liver, pale icteric skin, swollen pale kidney, and hemorrhage on the skeletal muscle. Histopathological examinations revealed large intranuclear inclusion bodies in hepatocytes, degeneration and congestion of liver sinusoids, and degeneration of renal tubules, spermatozoa, spermatid, Sertoli cells, and Leydig cells in the testicle. There were intertubular hemorrhages and large vacuoles. The seminiferous tubular lumens were dilated, contained necrotic debris, and were devoid of spermatozoa in the interstitial tissue. Lesions in the testicle are reported for the first time in the present study. RT-PCR was used to detect the virus by amplification of partial 1300 bp *hexon* genes. The amplified fragments were confirmed by sequencing. Our results concluded that two different genotypes circulate in Kurdistan, and the nucleotide sequence of Kurdistan fowl adenovirus (FAdV) isolates show only 81% homology together. The FAdV/Kurdistan/2013 and FAdV/Kurdistan/2020 belonged to FAdV-E close to USA isolates. On the other hand, the FAdV/Kurdistan/2015 belonged to FAdV-D closer to the Chinese FAdV isolate.

Keywords

Adenovirus, inclusion body hepatitis, broiler, histopathology, PCR

INTRODUCTION

Fowl adenoviruses (FAdVs) cause many diseases in chickens like inclusion body hepatitis (IBH), hydropericardium hepatitis syndrome (HHS), and adenoviral gizzard erosion (AGE), leading to economic losses everywhere in the world (Schachner, Matos, Grafl, & Hess, 2018).

Fowl adenoviruses (FAdVs) belong to the genus *Aviadenovirus*within the family Adenoviridae. They are non-enveloped double-stranded DNA viruses (De Luca et al., 2020). The International Committee on Taxonomy of Viruses separated Adenoviridae members into five genera (Zhao, Zhong, Zhao, Hu, & Zhang, 2015). The Mastadenovirus genus contains mammalian adenoviruses like humans, bats, dogs, horses, mice, -ruminants, and swine. The genus Aviadenovirus, formerly designated as a gaggle I avian adenoviruses (AAV), contains 11 of the 12 recognized European adenovirus serotypes classified into five molecular groups (A to E) and other related viruses (Hafez, 2011). Fowl adenovirus is immune to many several disinfectants and is comparatively tolerant to heat and pH changes. Iodophor and aldehyde disinfectants seem to be effective if they can contact the virus for an extended time (Rahimi & Haghighi, 2015).

The first IBH report was from the USA in 1963. Helmboldt and Frazier described the disease as necrotizing hepatitis in seven-week-old chickens (Helmboldt & Frazier, 1963). After that, the disease was reported in many areas. In 1988, a new broiler disease, called Angara Disease, was reported from Angara Goth near Karachi in Pakistan. The disease course and clinical signs were almost similar to IBH. The pathological findings included the accumulation of clear, straw-colored fluid in the pericardial sac; hence, it was called Hydropericardium Syndrome (Yasmeen et al., 2017). The disease has subsequently been recorded in Iraq (Abdul-Aziz & Al-Attar, 1991).

IBH is transmitted by vertical and horizontal means, but the former is reported as a very effective means of spreading from parent birds to offsprings (Asthana, Chandra, & Kumar, 2013). Horizontal infection occurs

through the oral-fecal route, and further spread by mechanical means and contamination with infected feces occurs (Gomis, Goodhope, Ojkic, & Willson, 2006).

Over the last two decades, increasing IBH outbreaks have been reported in several geographic locations, stressing the disease's worldwide spread. IBH mainly affects broilers aged up to 35 days, but the disease has also been described periodically in layers and broiler breeders. The disease has been reported in birds as young as seven day-olds and as old as 20 weeks (Erny, Barr, & Fahey, 1991). In natural outbreaks, IBH is characterized by sudden mortality of 2-40% in chickens. High death rates occur when the affected birds are younger than three weeks. Depending on the virus's pathogenicity, chicks' immune status, and simultaneous secondary infections, mortality as high as 80% may occur. In general, mortality peaks within 3–4 days and falls in 9–14 days (Schachner, Marek, Grafl, & Hess, 2016).

In most cases, the liver is the primarily affected organ. Gross lesions of IBH include an enlarged pale and friable liver, sometimes with necrotic foci. Ecchymotic hemorrhages might also be observed in the liver and muscles of the leg and breast. Clinical signs are not conclusive, including lethargy, huddling, ruffled feathers, and appetite loss (Hafez, 2011).

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 (Steer, O'Rourke, Ghorashi, & Noormohammadi, 2011). Histopathological lesions include the presence of focal necrotic areas and intra-nuclear inclusion bodies in some hepatocytes. The inclusion bodies might be eosinophilic, large, round, or irregularly shaped with a clear pale halo or occasionally basophilic (Schachner et al., 2018).

Most avian adenoviruses are considered opportunistic pathogens that do not produce clinical signs when inoculated into birds. However, researchers have noted that some viruses are considered primary pathogens, like those liable for inclusion body hepatitis, hydropericardium syndrome, respiratory disease, necrotizing pancreatitis, and adenoviral gizzard erosion in chickens and other birds. Moreover, infection with infectious bursal disease virus (IBDV) has been suggested as a significant predisposing factor in the development of IBH (Ojkic et al., 2008).

According to our knowledge, IBH or similar cases characterized by hepatitis and occurrence of hepatocytic intra-nuclear inclusion bodies have not been documented in poultry in Kurdistan of Iraq. Therefore, the case of adenovirus-like inclusion body hepatitis in a broiler farm in Kurdistan of Iraq is reported here for the first time.

METHODS

Field samples and clinical finding

The study was administered from April 2013 to May 2020, including 46 broiler farms from different Sulaymaniyah governorate areas, Kurdistan of Iraq. Clinically, the birds showed a sudden onset of high mortality, lethargy, ruffled feathers, and inappetence. At necropsy, the livers were the primarily affected organs, appearing as enlarged, pale yellow with necrotic foci and multiple petechial hemorrhages, enlargement of kidney, and hemorrhage in skeletal muscles. Tissue samples of liver, kidneys, and testicles were fixed in 10% neutral-buffered formalin solution for histopathology. For virus detection by RT-PCR also pool issue samples were collected from livers, kidneys, and spleens.

Histopathological examination

Tissue samples of liver, kidney, and testicles were fixed in 10% neutral-buffered formalin solution. Tissues were ordinarily processed and embedded in paraffin. Five-millimeter thick sections were fixated and stained with hematoxylin and eosin stains (Luna, 1968).

DNA extraction

Total DNA was extracted from 20–35 mg of pooled tissue samples consisting of liver, kidney, and spleen according to the manufacturer's instructions of a tissue DNA extraction kit (Bioneer, Korea).

PCR amplification

The PCR amplification reaction was accomplished in 0.2 mL tubes using AccuPower PCR PreMix. The reaction mixture consisted of 5 µL DNA, 1 µL (10 pmol) forward primer AACGTCAACCCCTTCAACTACC, and 1 µL (10 pmol) reverse primer TTGCCTGTGGCGAAAGGCG. The volume was then completed to 20 µL using DEPC-H_2O. The thermocycler was programmed to start with an initial denaturation at 94 °C for 5 minutes. Then, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56 °C for 30 seconds, and elongation at 72 °C for 90 seconds followed. The final 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 PCR products' sizes were estimated according to the migration pattern of a 100 bp DNA ladder. The PCR product was sequenced from both terminals by the Macrogen sequencing service in South Korea. The DNA sequence was published in GenBank as Fowl adenovirus isolate FAdV/Kurdistan/2013 accession number KF601576, FAdV/Kurdistan/2015 accession number KU060147, and FAdV/Kurdistan/2020 accession number MN967070.

Phylogenetic analysis

The partial *Hexon* gene sequence identity of all Kurdistan FAdV isolates was determined by the blast method at the National Center for Biotechnology Information (NCBI) homepage. A phylogenic tree was constructed with the VP2 hypervariable region of 20 FAdV isolates of different genotypes based on the neighbor-joining method using the Kimura2-parameter model Mega 7. The bootstrap values were determined 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 46 farms around Sulaymaniyah governorate in the Kurdistan of Iraq, and the clinical signs in 2–3 week-old broiler chicks included lethargy, huddling with ruffled feathers, inappetence, and yellow mucoid droppings.

The mortality rates ranged between 8% and 15%, and postmortem findings included pale icteric skin and ecchymotic hemorrhages on the skeletal muscles (Figure 1A). Enlarged, pale, and mottled liver (Figure 1B and 1C), swollen pale kidney with distended tubules (Figure 1D). The bursa of Fabricius and thymus had not atrophied. 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 (Figure 2), appearing as large basophilic intranuclear inclusion bodies surrounded by a pale halo. Many hepatocytes were degenerated and swollen with vacuolated cytoplasm. Infrequently, necrosis of scattered hepatocytes occurred. Moreover, there was lymphocytic and heterophilic hepatitis. These inflammatory infiltrates appeared clearly around the central vein. Additionally, there was widening and infiltration of sinusoids with lymphocytes, heterophils, and histiocytes (Figure 3).

Histopathological sections of testicles from infected birds revealed degeneration of spermatozoa, spermatids, Sertoli cells, and Leydig cells. In the interstitial tissue, there was intertubular hemorrhage and large vacuoles. Also, there was dilation of seminiferous tubular lumens that contained necrotic debris and were devoid of spermatozoa, a lesion reported for the first time in the present study (Figure 4).

Serotyping of FAdV isolates

Detection of fowl adenovirus was conducted using partial *hexone*genes of FAdV, and it was successfully amplified. The expected amplicon size was 1300 bp of FAdV, detected in three pooled samples from different

broiler farms. The amplified fragments were confirmed by sequencing (Figure 5). The nucleotide sequence of Kurdistan FAdV isolates showed only 81% homologies.

Phylogenetic analysis of FAdV isolates

A phylogenetic tree was constructed based on the partial hexon gene of FAdV sequence alignment of the 46 isolates (Figure 6). The FAdV isolates were distinctly divided into five general clusters: FAdV-A, FAdV-B, FAdV-C, FAdV-D, and FAdV-E. The phylogenic tree's topology indicated that two different FAdV genotypes with different genomic evolutions circulate in Kurdistan. The FAdV/Kurdistan/2013 and FAdV/Kurdistan/2020 belonged to FAdV-E close to USA isolates. On the other hand, the FAdV/Kurdistan/2015 belonged to FAdV-D closer to the Chinese FAdV isolate.

DISCUSSION

Iraqi Kurdistan contains more than 1300 poultry farms, and intensive farming and deficient control strategies have led to the spread of new viral infections. Thus, epidemiologic research is crucial to monitor disease outbreaks and develop vaccines. FAdV outbreaks have become a considerable concern for poultry farmers globally (Cizmecigil et al., 2020). IBH causes mortality resulting in production and economic losses. A higher number of FAdV clinical cases have been reported in recent years, and multiple FAdV strains have been isolated from sick birds in many countries (Mittal, Jindal, Tiwari, & Khokhar, 2014; Schachner et al., 2018). There is no published data about FAdV in Kurdistan chickens, reinforcing the requirement for molecular surveys on this emerging infectious agent in poultry and studying its fundamental role in clinical diseases. This study describes an IBH outbreak in Kurdistan/Iraq broiler flocks.

In this study, sudden high acute mortality of 8% to 15% started at two days to four weeks of age in affected broiler flocks. IBH can infect chickens of all ages; however, young chicks were more susceptible during the first two weeks, even when immunologically intact. There is an apparent age effect with avian adenoviruses. As the host's age increases, the multiplication of the viruses within the host is restricted, and mortality decreases (Rahimi & Haghighi, 2015). There was no accumulation of straw-colored fluid in the pericardial sac, indicating no relation with HHS. This outcome is probably because of the different viral strains and serotypes of FAdV causing infection in this study, which agrees with previous studies (El-Tholoth & Abou El-Azm, 2019; Mittal et al., 2014). Mortality during IBH outbreaks is generally between 2% and 10% of the flock, but up to 30% has been described in case of coinfection with other immunosuppressive causative agents (El-Tholoth & Abou El-Azm, 2019). Typical necropsy observations such as an enlarged and pale liver, enlarged and hemorrhagic spleen and kidneys, and clinical findings were detected in this study's IBH cases (Ahamad, Selvaraj, Sasikala, & BabuPrasath, 2016; Nakamura et al., 2011). Also, a pale and enlarged pancreas and greenish diarrhea were observed in some chicks, as reported by others (Ahamad et al., 2016).

The most critical histopathological finding in liver sections of diseased birds is the presence of intra-hepatocytic inclusion bodies. These are large basophilic intranuclear inclusion bodies surrounded by a pale halo (Anjum, Sabri, & Iqbal, 1989). Many hepatocytes were degenerated and appeared swollen with vacuolated cytoplasm. Infrequently, there was necrosis of some scattered hepatocytes. Moreover, there was lymphocytic and heterophilic hepatitis (Nakamura et al., 2011). These inflammatory infiltrates appear clearly around the central vein. Additionally, there was widening and infiltration of sinusoids with lymphocytes, heterophils, and histiocytes.

Histopathological sections of testicles from infected birds revealed degeneration of spermatozoa, spermatids, Sertoli cells, and Leydig cells. In the interstitial tissue, there was intertubular hemorrhage and large vacuoles. Also, there was dilation of the seminiferous tubular lumens containing necrotic debris and devoid of spermatozoa. Lesions in the testicle are reported for the first time in the present study.

In the present study, the isolates' phylogenetic analysis revealed the circulation of two different genotypes in Kurdistan. Both Kurdistan FAdV had different genomic evolution. The FAdV/Kurdistan/2013 and FAdV/Kurdistan/2020 belonged to FAdV-E close to USA isolates. On the other hand, the FAdV/Kurdistan/2015 was closer to the Chinese FAdV isolate, proposing possible virus spreading from

these areas, perhaps through trades and primary breeders or breeder substitutes. Circulation of two genotypes of FAdV in Kurdistan may hurt the clinical disease because some strains of different serotypes can reproduce IBH and hydropericardium syndrome simultaneously (Zhao et al., 2015).

Immunosuppressors such as IBDV, chicken infectious anemia virus (CIAV), and Marek's disease virus (MDV) are factors that facilitate IBH outbreaks propagation or worsen clinical manifestations of FAdV infections (Morshed, Hosseini, Langeroudi, Fard, & Charkhkar, 2017; Niczyporuk, Woźniakowski, Samorek-Salamonowicz, & Czekaj, 2013). However, several studies showed that IBH might occur as a primary disease (Gomis et al., 2006; Ojkic et al., 2008).

CONCLUSION

This study is the first molecular characterization and histopathological examination of FAdVs in Sulaymaniyah broiler farms. FAdVs could be emerging infectious agents in Kurdistan poultry flocks, causing severe disease in young chicks. More research is needed to evaluate fowl adenoviral serotypes' prevalence and pathogenicity in poultry flocks in Iraq. Identification of FAdV serotypes is essential in epidemiological studies of the disease outbreaks, development of preventative measures, and adoption of vaccination strategies. There is also a need for future development in molecular methods to identify adenovirus strains' origins in IBH.

ACKNOWLEDGMENTS

The authors wish to thank Micro Vet Diagnostic Laboratory for providing sample materials and contributing valuable information to the relevant cases and the College of Veterinary Medicine/University of Sulaimani for funding this study.

ETHICAL APPROVAL

The research was conducted following the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

DATA AVAILABILITY STATEMENT

The data that support the findings are available on request from the corresponding author.

CONFLICT OF INTEREST STATEMENT

The authors report no potential conflict of interest.

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FIGURE LEGENDS

Figure 1. Gross lesions in infected broilers with fowl adenovirus. A: ecchymotic hemorrhages in the skeletal muscles, B: Hepatomegaly (right) compared to the normal liver (left), 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.

Figure 2. The histopathological section in the liver. A: Moderate lymphocytic and heterophilic inflammatory infiltrates surrounding the central vein. Diffuse cytoplasmic vacuolation is observed within remaining hepatocytes, and there is widening and infiltration of sinusoids with lymphocytes, heterophils, and histiocytes. B: In the previous slide with more magnification, the nuclei of numerous hepatocytes contained one large basophilic inclusion body (arrow), a halo is present around the intranuclear inclusion, and the nucleus membrane was hyperchromatic (H and E stain, X100, X200).

Figure 3. Histopathological sections in the liver. A: Several individual necrotic hepatocytes are scattered within the parenchyma. There is hepatic parenchymal disruption due to coalescing of randomly distributed foci of degenerated hepatocytes. These hepatocytes are swollen with hypereosinophilic and highly vacuolated cytoplasm (H and E stain, X100). (B) Numerous hepatocyte nuclei contained one large basophilic inclusion body as indicated by white arrows (H and E stain, X100). C: Lymphocytic and heterophilic infiltration surrounding and close to a central vein. Diffuse cytoplasmic vacuolation is observed within remaining hepatocytes. Additionally, there is widening and infiltration of sinusoids with lymphocytes, heterophils, and histiocytes (H and E stain, X200). D: Large basophilic intranuclear inclusion bodies (white arrows). The presence of free RBC is indicated by yellow arrows (H and E stain, X400).

Figure 4. Histopathological sections in the testicle. A: Degeneration of spermatozoa, spermatid, Sertoli cells, and Leydig cells. In the interstitial tissue, there is intertubular hemorrhage (H) and large vacuoles (arrows). B: Dilation of seminiferous tubular lumens containing necrotic debris and devoid of spermatozoa in some of them (inset). H and E, X100.

Figure 5. Agarose gel electrophoresis pattern shows PCR amplification of 1300 bp from *hexon* gene of Kurdistan FAdV Lane L: 100 bp DNA ladder. Lane 1 indicates FAV field strain.

Figure 6. Phylogenetic tree of Kurdistan FAdV isolates. Analysis of the phylogenetic tree according to partial *Hexon* gene sequence indicates five clusters. The red circle indicates FAdV/Kurdistan/2013 and FAdV/Kurdistan/2020. The pink circle indicates FAdV/Kurdistan/2015.