ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (1): 19 - 25
doi: 10.30466/vrf.2024.2030590.4302
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Molecular and histopathological characterization of fowl adenovirus serotype 4 in broiler chickens in Kashan, Iran: implications for disease control and prevention in the poultry industry

Mohammadreza Ghorani[1]*, Amir Ali Shahbazfar[1], Mohsen Ghorbiani[2], Behzad Ghorbanzadeh[3], Rohollah Kamyabi[4]

[1] Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran; [2] Private Avian Clinic, Kashan, Iran; [3] Central Laboratory of Veterinary Science, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran; [4] Department of Molecular Veterinary Directorate General of Tehran Province, Tehran, Iran.

Article Info

Article history:

Received: 23 June 2024
Accepted: 06 October 2024
Available online: 15 January 2025

Keywords:

Broiler chickens
Epidemiology
Fowl adenovirus
Hydropericardium-hepatitis syndrome
Phylogenetic analysis

Abstract

Fowl adenovirus (FAdV) is a DNA virus causing significant diseases, like inclusion body hepatitis, hydropericardium-hepatitis syndrome (HHS), and gizzard erosion. These diseases lead to severe economic losses in the poultry industry. Recent increases in HHS outbreaks in Iran, particularly among broilers, prompted this study to analyze FAdV isolates in Kashan, Iran. In December 2021, a high-mortality HHS outbreak in a Kashan broiler flock led to liver and heart samples being sent for analysis. Histopathological investigations revealed mononuclear hepatitis and intra-nuclear viral inclusion bodies in hepatocytes. Polymerase chain reaction and phylogenetic analyses confirmed the presence of FAdV-4 (accession number: PP856395), showing 99.99% identity with strains from Japan, the United Arab Emirates, Pakistan, and the United States. These findings highlight the genetic similarity and potential common origin of FAdV-4 strains. This study emphasizes the need for heightened biosecurity measures and effective vaccination strategies to mitigate the spread of FAdV-4. The confirmed presence of FAdV-4 in central Iran poses a significant threat to the poultry industry, necessitating prompt action to prevent substantial economic losses.

Introduction

Fowl adenovirus (FAdV) is a DNA virus belonging to the Adenoviridae family and the Aviadenovirus genus. This virus is classified into five species (FAdV-A through FAdV-E), including 12 serotypes (FAdV-1 to 8a, and 8b to 11). The FAdVs can infect chickens, ducks, peacocks, pigeons, and wild birds, leading to severe clinical symptoms.[1-8] Diseases, such as inclusion body hepatitis (IBH), hydro-pericardium-hepatitis syndrome (HHS), and adenoviral gizzard erosion (AGE) are associated with FAdV infections. The widespread occurrence of FAdV has resulted in significant economic losses in the poultry industry due to these diseases.

In 1987, the HHS, linked to the serotype 4 fowl adenovirus (FAdV-4), was discovered and reported in the Angara Goth province of Afghanistan. This condition is also known as Angara disease.[9] Subsequently, it was reported in India, China, South Korea, Japan, Canada, and the United States, leading to significant economic losses in the global poultry industry.[3,10-16] Since June 2015, a natural outbreak of severe HHS associated with a hypervirulent new genotype of FAdV-4 has occurred in most provinces of China.[3,11] The new FAdV-4 genotype spreads rapidly, resulting in a mortality rate of 30.00 - 100%, significant economic losses, and serious threats to the poultry breeding industry. Additionally, the detrimental effects of this disease are exacerbated when FAdV-4 co-infects with another virus.[17-19] In recent years, the number of HHS outbreaks across various regions of Iran has risen, particularly among broiler flocks.[20,21] This study presents the molecular analysis of FAdV isolates and histopathological examinations conducted in Kashan, Iran.

Materials and Methods

Sampling. In December 2021, a broiler flock (Ross 308) in Kashan, central Iran, experienced high mortality

***Correspondence:**

Mohammadreza Ghorani. DVM, PhD
Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
E-mail: ghorani@tabrizu.ac.ir

due to HHS. This flock of 26,000 broiler chickens began showing mortality at the age of 11 days, with daily losses reaching 10.00%. Liver and heart samples (20 numbers from each tissue randomly) were kept on dry ice under sterile conditions and submitted to the Veterinary Virology Laboratory in Tabriz, Iran, where FAdV was detected. For histopathological studies, the samples were placed in 10.00% formalin and referred to the Pathology Laboratory of the Faculty of Veterinary Medicine, Tabriz University, Tabriz, Iran. There was not another poultry farm around this infected farm. None of the chickens had been vaccinated against adenoviruses.

Histopathological investigations. Tissue specimens were collected from the livers of 13 birds from various locations and placed in 10.00% neutral buffered formalin. After 24 hr, the formalin solution was replaced. Following 1 week of fixation, the liver specimens were processed using a tissue processor (Shandon Citadel 2000; Thermo Fisher Scientific, Waltham, USA) with xylene (Dr. Mojallali Pharmaceutical Chemicals Industries Complex Co., Saveh, Iran) and increasing ethanol concentrations and then, embedded in paraffin. The specimens were sectioned at a thickness of 5.00 µm using a microtome (Leica, Wetzlar, Germany), and microscopic slides were prepared. The slides were stained with Hematoxylin and Eosin and examined under a light microscope (ML2100; A. Kruss, Hamburg, Germany).

Molecular test. All tissue samples were combined and homogenized in a sterile porcelain mortar. After thorough mixing, physiological serum was added to the homogenized solution. Viral DNA was extracted from this solution using a SinaPure Viral Kit (SinaClon, Tehran, Iran) following the manufacturer's instructions. To detect fowl adenovirus, specific primer sequences were used. The forward primer, Hex L1-s (sequence: ATGGGAGCSACCTAY TTCGACAT), and the reverse primer, Hex L1-as (sequence: AAATTGTCCCKRAANCCGATGTA), with a product of 587 base pairs (bp) based on the *hexon* gene.[1] Polymerase chain reaction (PCR) mixture was prepared by combining 4.00 µL dNTP (1.25 mM), 1.00 µL of each of forward and reverse primers (25.00 µM), 5.00 µL PCR buffer (5.00 X), 1.25 U DNA polymerase enzyme (SinaClon), 2.00 µL viral DNA, 2.00 µL $MgCl_2$ (25.00 mM), and nuclease-free water to a final volume of 25.00 µL. The positive control from the authors' previous study was included in this research.[22] A negative control sample consisting of distilled water was also included. The amplification was done using the Thermal Cycler (T100, Bio-Rad, California, USA) following the program outlined in Table 1. This process involved 40 cycles from steps two to four. The PCR products were then analyzed by electrophoresis in 1.50% agarose gels stained with safe stain and visualized using the ultra-violet transilluminator. The purification of PCR products was conducted using AccuPrep® Purification Kit (Bioneer, Daejeon, South Korea). Sequencing in both the forward and reverse directions was carried out using ABI genetic analyzer (3500; Applied Biosystem, Waltham, USA).

Phylogenetic analysis. The sequence alignment and phylogenetic analysis based on the neighbor-joining method were performed using CLC Sequence Viewer 8.0.0 (Qiagen, Aarhus, Denmark). Finally, the nucleotide sequences of the *hexon* gene in the samples were compared with those of other FAdV strains registered in GenBank®.

Table 1. Polymerase chain reaction thermal cycle program.

Times	Temperature (˚C)	Step
2 min	94.00	1
20 sec	94.00	2
20 sec	56.00	3
30 sec	72.00	4
2 min	72.00	5

Results

At necropsy, the liver was enlarged, yellowish, and sometimes pale. Hydropericardium was also observed in the heart (Fig. 1).

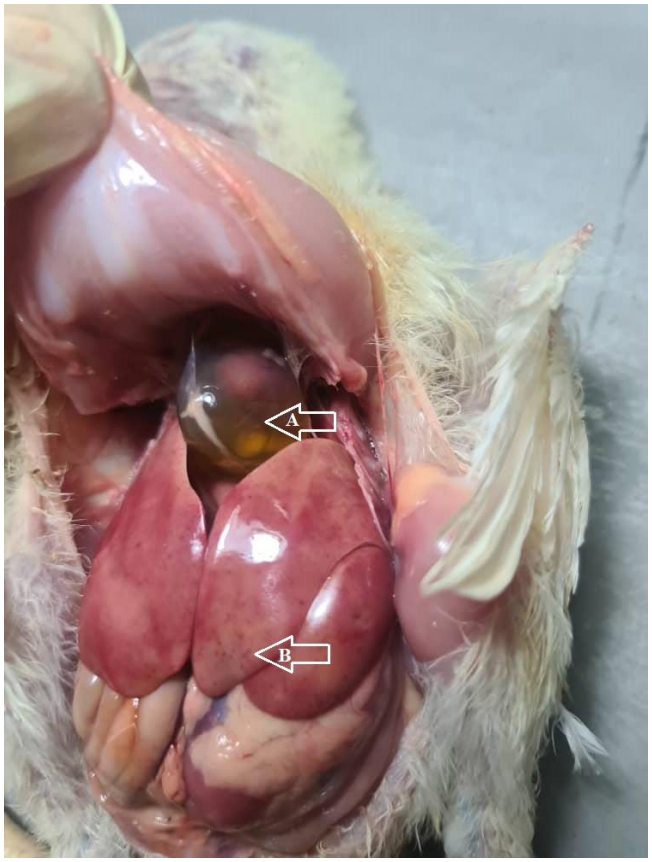

Fig. 1. A broiler chicken died due to the fowl adenovirus. A indicates the hydropericardium in the heart; B shows the petechial hemorrhages and diffused necrosis within enlarged and yellowish liver.

The PCR analysis detected FAdV infection in both liver and hydropericardium-heart samples, with specific bands visualized *via* gel electrophoresis (Fig. 2).

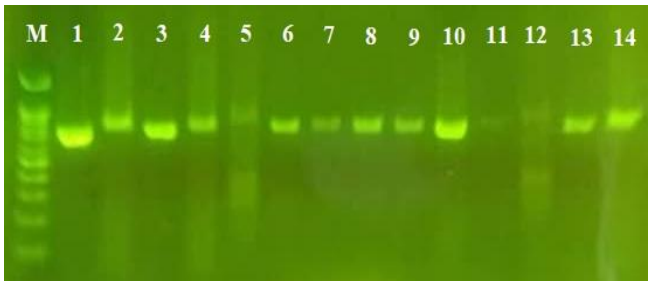

Fig. 2. Agarose gel electrophoresis of polymerase chain reaction products. The lanes illustrate molecular weight marker of 50 bp (M), positive control (1), positive samples with the 587 bp band (2, 3, 4, 6, 7, 8, 9, 10, 13, and 14), negative samples (5 and 12), and negative control (11).

Additionally, slight hyperemia was noted in the liver. Histopathological examination revealed focal primarily mononuclear hepatitis in the liver (Fig. 3A). Furthermore, intra-nuclear viral inclusion bodies with chromatin margination were observed in some hepatocytes (Figs. 3B and C). Therefore, it was concluded that the chickens were infected with adenovirus.

Phylogenetic analysis confirmed that the strain analyzed in this study (accession number: PP856395) belongs to the same group as strains LC504494 from Japan, OL456287 from the United Arab Emirates, OR351954 and OQ291173 from Pakistan, and FR686931 from the United States, with 99.99% identity (Fig. 4). All these strains are classified as FAdV-C serotype 4.

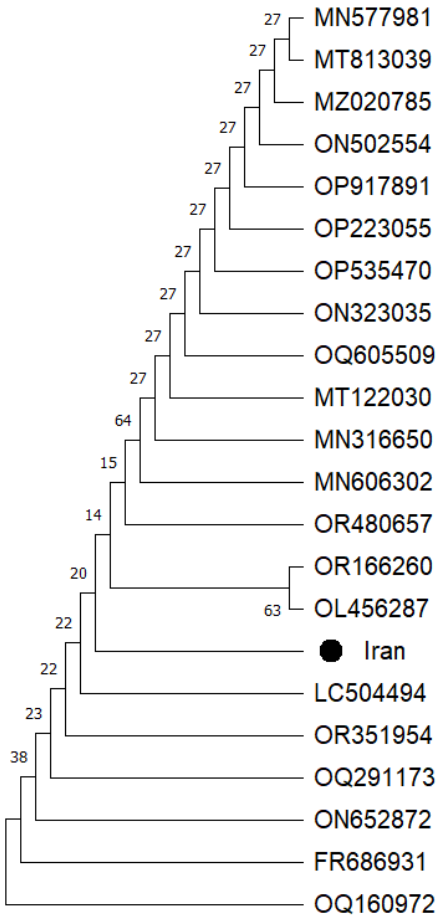

Fig. 4. Fowl adenovirus phylogenetic tree constructed based on *hexogen* gene with MEGA (11.0.13).

Discussion

Fowl adenoviruses are classified into five types (A to E) based on their genetic characteristics. In China, Chinese virologists isolated a highly virulent strain of FAdV-4 (hvFAdV-4) from 25-day-old broilers with severe HHS in Guangdong Province. Wang *et al.* also detected five FAdV-4s isolated from eastern China.[23-25]

To identify and investigate FAdV serotypes associated with IBH or HHS in commercial poultry in certain regions of China from 2007 to 2017, Wang *et al.*, conducted a study. They isolated eighty FAdV strains from liver and kidney samples of the diseased poultry. Serotype 4 was found in 53.70% of the strains and linked to the wide-spread use of adenovirus vaccines containing inactivated serotype 4. These findings indicated that between 2007

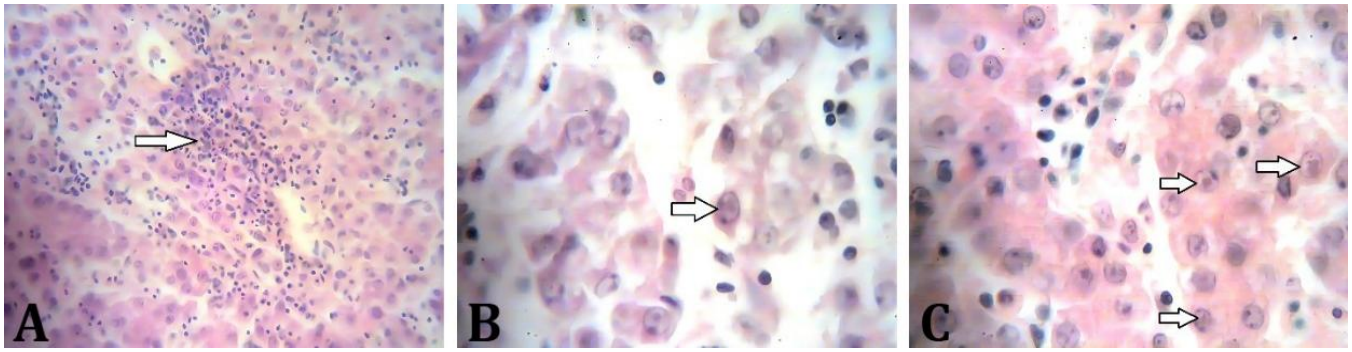

Fig 3. Histopathological analysis of liver infected with the fowl adenovirus using Hematoxylin and Eosin staining. **A)** Infiltration of mononuclear cells. Arrow shows focal inflammation in the liver (200×); **B)** Nuclear chromatin margination. Arrow shows intra-nuclear viral inclusion body (2,000×); and **C)** Nuclear chromatin margination. Arrow shows intra-nuclear viral inclusion bodies (2,000×).

and 2014, the primary serotypes in certain Chinese regions were FAdVs 11, 4, and 8b. Layers were the predominant hosts infected with serotype 4 of chicken adenovirus, which could also be infected with serotype 11.[26] In Japan, Mase *et al.*, studied the genetic characterization of FAdV-4 isolated from chickens with HHS.[14] In Pakistan, a field isolate of adeno-associated virus serotype 4 (AAV-4) obtained from an Angara disease outbreak was isolated through growth in chick embryo liver cells. The PCR was then utilized to amplify the hypervariable region of the *hexon* gene of AAV-4.[27] Fifty field samples were collected in the Shamim *et al.,* study, but only six were chosen for molecular analysis. The hydropericardium syndrome isolates were confirmed to be FAdV type 4 through PCR.[28] The report from Poland indicated the isolation of 96 field strains of FAdV from chickens. A PCR assay targeting specific L1 loop regions of the *hexon* gene was conducted. These isolates were classified into five species of FAdV (A to E), representing eight serotypes based on the sequences of the adenovirus strains.[29]

Diseases commonly caused by FAdVs include HHS, IBH, AGE, lung disorders, and hemorrhages in the muscles and organs. In Iran, the most prevalent disease associated with FAdV is IBH, accounting for over 70.00% of cases. Following this, AGE is the next most common disease. Former studies also revealed that serotypes 11 and 8b and genotypes D and E are frequently linked to the IBH in Iran. Furthermore, FAdV-1 was identified as the most commonly isolated serotype associated with AGE disease in Iran. Additionally, based on previous research findings, FAdV-4 has been identified as a primary serotype linked to HHS isolates in Iran.[30]

Vertical transmission of FAdV has been observed, indicating that the virus can be passed from parent birds to their offspring.[31] Vertical transmission of FAdV could potentially contribute to the virus's rapid spread in Iran. While strict biosecurity measures during the rearing period help prevent the introduction of FAdVs, there is still a risk of infection during the production phase, potentially leading to airborne transmission. The ability of FAdVs to spread vertically to offspring suggests a mechanism for inter-flock transmission. In September 2019, a highly infectious disease affected a peacock farm in central China, resulting in severe health issues. The disease exhibited a high mortality rate of 78.60% in peacocks aged 28 to 42 days. This study isolated a strain of highly pathogenic FAdV-4 from the peacocks.[32] To investigate epidemiological changes and the characteristics of the virus before and after the introduction of various commercial FAdV-4 vaccines, a total of 146 FAdV strains were collected and analyzed in South Korea from 2013 to 2019. The findings of these studies indicated a recent shift in the dominant serotype of FAdV from FAdV-4 to FAdV-8b. Specifically, in 2019, FAdV-8b accounted for 77.80% of the serotypes identified in field cases, whereas FAdV-4 comprised only 18.50%.[33]

The first occurrence of HHS in Iranian broiler chicken flocks was reported by Toroghi *et al.*, when examining the possibility of HHS in slaughtered broilers.[34] According to the earlier study, FAdV-4 was identified as a primary serotype associated with HHS isolates in Iran.[30] Previously, HHS cases in Iran were not given significant attention. However, there has been a noticeable increase in the number of HHS cases over time. The FAdVs were isolated from the northern provinces of Iran in 2013 and 2014 and subsequently, detected in the central regions of Iran in 2015.[34] Khabiri *et al.*, have submitted the complete genomic sequence of the fowl adenovirus C (FAdV-4) strain FAdV-4/Pasouk to the GenBank®. This strain was isolated from chickens suffering from HHS during an outbreak in Iran. The FAdV-4 isolate exhibited substantial genetic resemblances to recent isolates from China, indicating a potential common origin.[35] The prevalence of FAdV disease is higher in the northern and central provinces of Iran.[30]

This study also determined that in Iran, as in other regions of the world, FAdV-4 is the main serotype associated with HHS. In this study, phylogenetic analysis of the isolate revealed that it belongs to FAdV species C and is associated with HHS, contrasting with other reports in Iran.[20,21] Sequences of FAdV-4 obtained in this study exhibited nucleotide identities ranging from 98.77 to 99.99% with each other (Table 2). The highest similarity was observed between the Iran strain and isolates from Japan, the United Arab Emirates, Pakistan, and the United States, with accession numbers LC504494, OL456287, OR351954, and FR686931, respectively. The close relationship of the strain of this study with the strains of Pakistan and the United Arab Emirates, which are near Iran, doubles the importance of monitoring the virus for veterinary organizations. Hence, basic and correct border control and quarantine are very important in disease prevention and control. There is an urgent need to effectively monitor FAdV in slaughter-aged chickens across the country. Molecular techniques and virus isolation methods can also be used to identify and classify field isolates. The only effective strategy to control this disease is to vaccinate broiler flocks in advance. It is also recommended to adhere to the regular vaccination schedules and use potent vaccines. Biosecurity at the level of poultry farms is one of the necessary measures for disease control. The lowest identity of the Iran FAdV was with isolate OQ160972 from Azerbaijan. Infectious bursal disease virus (IBDV) and avian infectious anemia virus (CIAV), known as immunosuppressive agents in birds, are predisposing factors for the outbreak of HHS or can exacerbate the clinical presentation alongside FAdV infections.[36,37] Studies conducted in Canada, the United States, and New Zealand have indicated that FAdVs can act

Table 2. Percentage of the *hexon* gene nucleotide sequence identity between Iran strain and reference strains.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1																					
2	99.99																				
3	99.99	100																			
4	99.99	100	100																		
5	99.99	100	100	100																	
6	99.99	100	100	100	100																
7	99.98	100	100	100	100	100															
8	98.77	98.80	98.80	98.80	98.80	98.80	98.78														
9	99.98	100	100	100	100	100	99.99	98.79													
10	99.98	100	100	100	100	100	99.99	98.79	100												
11	99.98	100	100	100	100	100	99.99	98.79	100	100											
12	99.98	100	100	100	100	100	99.99	98.79	100	100	100										
13	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100									
14	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100								
15	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100							
16	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100						
17	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100	100					
18	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100	100	100				
19	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100	100	100	100			
20	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100	100	100	100	100		
21	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100	100	100	100	100	100	
22	99.98	100	100	100	100	100	99.99	98.79	100	100	100	100	100	100	100	100	100	100	100	100	100

1: Iran; 2: LC504494; 3: OR351954; 4: OQ291173; 5: ON652872; 6: FR686931; 7: OR480657; 8: OQ160972; 9: MN316650; 10: MT122030; 11: OR166260; 12: OQ605509; 13: ON323035; 14: OP535470; 15: OP223055; 16: OP917891; 17: ON502554; 18: OL456287; 19: MZ020785; 20: MN577981; 21: MT813039; 22: MN606302.

as a primary disease without being associated with IBDV or CIAV.[31,38-41] In Iran, the vaccination of poultry breeding flocks with virulent anemia and respiratory bursa virus vaccines leads to the production of sufficient antibodies in chickens (from maternal sources). This provides adequate protection against both clinical and subclinical forms of the disease at an early age. Additionally, some flocks infected with HHS did not show detection of CIAV and IBDV through molecular tests. According to the published data by Hosseini and Morshed, FAdVs are the most common primary disease in Iran and require increased attention.[21]

Identifying FAdV serotypes is crucial for conducting epidemiological studies on outbreaks, developing prevention measures, and implementing vaccination strategies. Nucleotide sequence diversity analysis serves as a reliable method of molecular epidemiology for characterizing FAdVs. The current study confirmed the presence of FAdV-4 in central Iran based on histo-pathological and molecular evidence. In recent years, there appears to be an increase in FAdVs among broiler chickens in Iran, posing a significant threat to the poultry industry, and potentially resulting in substantial losses.

Acknowledgments

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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