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Original Article

Comparison of Penton and Hexon Genes of Fowl Adenovirus Serotype 11 in Molecular Detection of IBH in Broilers

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Abstract

Fowl Adenoviruses (FAdVs) are widely distributed pathogens across the globe. The FAdVs from serotypes FAdV 2, 3, 8a, 8b, 9, and 11 are responsible for inclusion body hepatitis (IBH). Recently, increased mortality and IBH-suspected lesions were observed in 8-10-day-old broiler chickens in West Azerbaijan Province, Iran. In this regard, the present study aimed to compare penton and hexon genes of ADDV11 in the molecular detection of IBH in broiler chickens. In total, 100 liver specimens were collected from 10 suspected farms, and their DNAs were extracted. Two polymerase chain reactions (PCRs) were applied; one targeting the L1 region of the hexon gene and another aiming at the penton gene. Based on the findings, 60% of samples showed positive results in both PCRs and phylogenetic analysis clustered the studied viruses into serotype 11 (species D) FAdV. The detected FAdVs also shared a multitude of homologies with previously published serotype 11 viruses from Iran and those identified in Pakistan, Saudi Arabia, India, China, and Canada. This research not only provides an update on circulating FAdVs in Iran, but also introduces the penton gene as an alternative target for IBH diagnosis. Considering that IBH is a primary disease in Iran with both horizontal and vertical routes of transmission, urgent preventive measures are needed.

Keywords: Broiler, Hexon gene, Inclusion Body Hepatitis, Iran, Penton gene

1. Introduction

Fowl adenoviruses (FAdVs) belong to the *Rowavirales* order, *Adenoviridae* family, and *Aviadenovirus* genus. Diseases caused by FAdVs can be presented by a variety of clinical manifestations that have turned it into a growing concern for the poultry industry around the world (1). This family includes very heterogenous groups of viruses that are classified into 12 types (previously known as serotype), named FAdV-1 to 8a and 5 species (A-E) based on the digestion pattern of restriction enzymes and serum cross-neutralization test (2). Infection with fowl adenoviruses can lead to different syndromes, like inclusion body hepatitis (IBH) syndrome, hydropericardium hepatitis syndrome (HHS), and gizzard erosions (3).

The IBH is mainly caused by serotypes FAdV-2, FAdV-9, and FAdV-11 which belong to species *Fowl adenovirus* D, serotype FAdV-3 which belongs to species Fowl adenovirus C, and serotypes FAdV8a and FAdV8b that belong to species *Fowl adenovirus* E (3-5). This syndrome was reported for the first time by Helmboldt and Frazier in 1963, and they described “an acute hepatic catastrophe” due to severe damage to liver tissue (6).

Two other important syndromes manifested by *Fowl adenoviruses* are hepatitis-hydropericardium syndrome and gizzard erosions caused by FAdV1 and FAdV4,

respectively (3). In 1987, HHS was reported for the first time in Pakistan, which was followed by India, Kuwait, Iraq, Japan, Korea, Russia, Mexico, and south and central America (7). Natural cases of gizzard erosions related to fowl adenovirus in chickens were first reported in 1993 and erosions induced by FAdV-1 were also reported in countries, such as the United Kingdom, Italy, Poland, Deutschland, Japan, and Korea (8).

The FAdVs are naked virions, harboring a double-stranded DNA genome sizing between 43 and 46 kbp, which encodes a few structural and non-structural proteins. The capsid is formed by three main structural proteins, involving 240 hexons, 12 penton bases at vertices, and 1 or 2 fibers protruding from each penton base (9, 10).

Hexon L1 region is genotype-specific and used for the characterization of all FAdVs serotypes. Considering the hexon L1 region, FAdV-1 and FAdV-5 are the sole members of FAdV species A and B, respectively. Moreover, FAdV-4 and FAdV-10 belong to species C while FAdV-2, FAdV-3, FAdV-9, and FAdV-11 exist in species D, and FAdV-6, FAdV-7, FAdV-8a, and FAdV-8b belong to species E (11).

The IBH is characterized by a sudden increase in mortality rate (10-30%) that peaks after 3-4 days and ceases on day 5; however, its morbidity rate is low (12). Classic type of IBH affects chickens up to 5 weeks old while the mortality rate during IBH outbreaks varies from 10% to 30%; this mortality rate and poor production have significant economic impacts (13). According to recent studies performed in Iran, the number of IBH cases is growing especially in broiler and broiler breeder flocks (12). To determine the FAdV types in Iran, the present study aimed to perform molecular characterization of FAdVs involved in IBH-suspected outbreaks. Apart from the analysis of the hexon L1 region, the penton gene was also analyzed to provide more comprehensive information. To the best of our knowledge, this is the first study that utilized two genes simultaneously for the detection of FAdV-11 among two-week-old chickens in Iran.

2. Materials and Methods

2.1. Sample Collection

2.1.1. Chickens

In 2021, an increased mortality rate was observed in 8-10 days old broiler chickens in West Azerbaijan province, Iran. Some chicks presented signs of depression and anorexia and a necropsy examination revealed pale and swollen livers with petechial hemorrhages. An investigation was carried out to assess the presence of IBH disease.

2.1.2. Sampling

In this study, 10 farms of broiler chickens in West Azerbaijan were selected and 10 liver specimens were collected from each farm (10 pooled samples from each farm). Samples were stored in dry containers and transported to the laboratory of the University of Tehran, Tehran, Iran. It is worth mentioning that the chicks did not receive any vaccine against fowl adenoviruses.

2.2. Histological Examination

For histopathological observation, liver samples were obtained from the suspected chickens (those with swelling, diffuse or focal paleness, and petechiae in the liver) and processed for histopathological examination according to standard methods. Briefly, the samples were fixed in 10% buffered formalin and embedded in paraffin. Sections were performed by microtome, stained with Hematoxylin and Eosin, and then examined under light microscope for histopathological changes.

2.3. Molecular Detection

2.3.1. DNA Extraction

Tissue specimens from the liver were chopped into small parts and homogenized, and the supernatant was isolated after being centrifuged. The viral DNA was extracted using the Sinaclon DNA extraction kit (Sinaclon, Iran).

2.3.2. Polymerase Chain Reaction and Primers

A polymerase chain reaction (PCR) amplifying a 590 bp region of the hexon gene was applied to confirm the presence of viral DNA. One set of primers was employed to amplify the Hex Loop1 (L1) gene, with the forward sequence of 5’-

ATGGGAGCSACCTAYTTCGACAT-3' and reverse primer of 5'- AAATTGTCCCKRAANCCGATGTA-3'. Additionally, a PCR targeting the penton gene was conducted according to a previously designed method with the expected size of 1406 bp (14).

2.3.3. Sequencing and Phylogenic Analysis

Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Bioneer Co., Korea) in forward and reverse directions. The L1 Hexon and penton gene sequences of our viruses, along with other FAdVs retrieved from the GenBank, were aligned by the ClustalW algorithm. Distance matrices were calculated using the Kimura 2-parameter model implemented in MEGA software (version 7.0.26). Neighbor-joining trees were plotted with MEGA software with a 1,000-fold bootstrap approach.

3. Results

3.1. Histological Examination

Histopathological examination of the livers collected from the suspected chickens revealed hyperemia and necrosis and the presence of basophilic intra-nuclear inclusion body in the hepatocyte cells (Figure 1).

3.2. Detection and Phylogenetic Analysis of the Hexon Gene

The primers successfully amplified the expected 590 bp DNA 6 out of the specimens submitted to this study. Six samples were sequenced and phylogenetic analysis of the partial hexon gene clustered the virus into serotype 11 and species FAdV-D. The comparison of obtained sequences with those retrieved from the GenBank revealed that almost all detected viruses shared a multitude of homologies with serotype 11 viruses reported in 2016 and 2013 in Iran and in 2018 in Pakistan. In addition, FAdV_D_URTE-3 also showed similar homologies to those of FAdV-11 viruses found in India and Saudi Arabia. High levels of similarity were also found between Favd_D_URTE-2 and those identified in China and Canada (Figure 2 and Table 1).

3.3. Detection and Phylogenetic Analysis of the Penton Gene

In total, 6 out of 10 samples showed positive results in the PCR targeting the 1406 bp penton gene. All of the positive results were subjected to sequencing and they showed close relationships with serotype 11 FAdV. Almost all of the strains analyzed in this study shared many homologies with FAdVs collected from Pakistan, Austria, the USA, the UK, and Switzerland. The penton gene sequences of UTRE-4, -5, and -6 were completely identical; moreover, they shared 100% of homologies with FAdV detected in 2018 in Pakistan. The UTRE-3 was also completely identical to those of FAdV-D viruses from China and Canada (Figure 3 and Table 2).

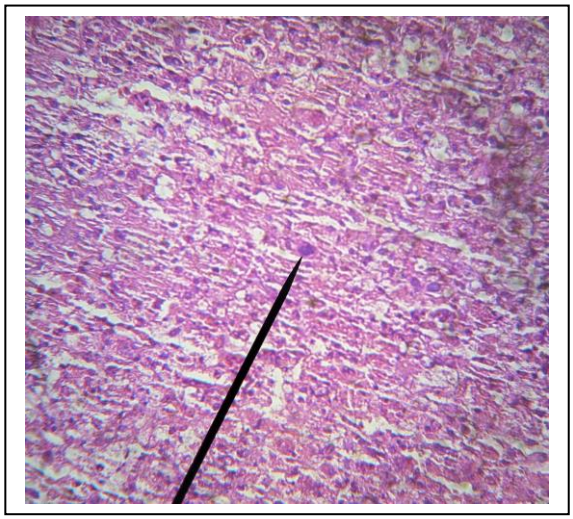

Figure 1. Microscopic view of the liver with basophilic intranuclear inclusion body and hyperemia and necrosis in the hepatocyte cells. (Hematoxylin and Eosin stain, 400X)

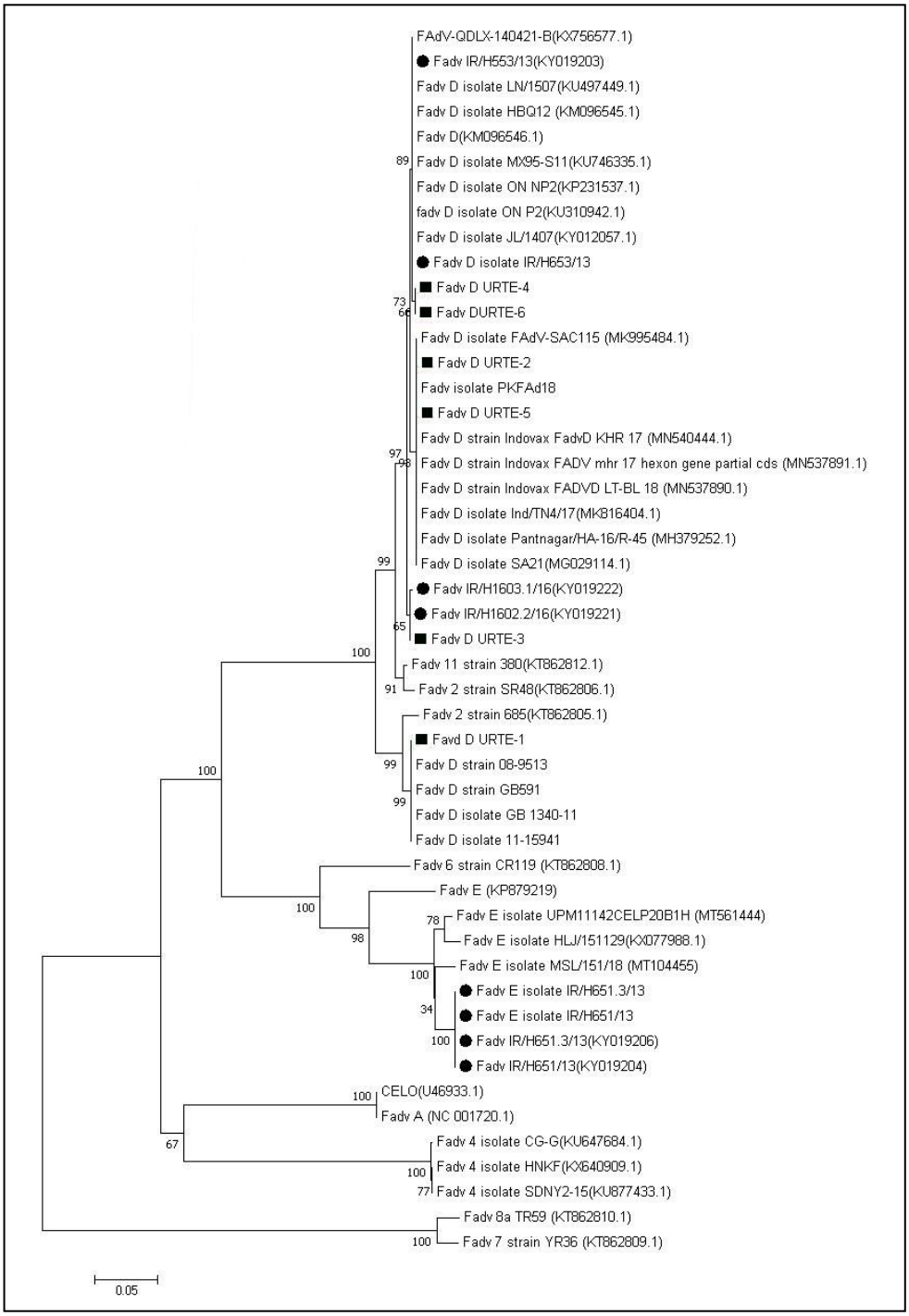

Figure 2. Phylogenetic tree based on the L1 loop of the hexon gene of fowl adenoviruses. Fowl adenoviruses detected in this study are shown with green squares, previously published Iranian strains are shown with black circles.

Table 1. Sequence identity matrix for FAdV-D viruses detected in this study with other related fowl adenoviruses, based on the loop region of the hexon gene

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	Favd_D_URTE-1																							
2	Fadv_D_URTE-2	91.20																						
3	Fadv_D_URTE-3	92.04	97.93																					
4	Fadv_D_URTE-4	89.95	96.78	95.79																				
5	Fadv_D_URTE-5	90.81	98.75	97.07	95.80																			
6	Fadv_DURTE-6	90.96	97.48	96.85	96.30	97.00																		
7	Fadv_D_strain_GB591	98.73	91.71	93.97	91.71	92.43	91.49																	
8	Fadv_D_isolate_GB_1340-11	98.73	91.71	93.97	91.71	92.43	91.49	100.00																
9	Fadv_isolate_PKFAd18	92.39	98.01	99.25	96.75	98.38	96.37	93.85	93.85															
10	Fadv_IR/H553/13(KY019203)	92.22	97.93	99.08	96.70	97.25	97.59	93.97	93.97	99.25														
11	Fadv_D_isolate_IR/H653/13	92.22	97.93	99.08	96.70	97.25	97.59	93.97	93.97	99.25	100.00													
12	fadv_D_isolate_ON_P2(KU310942.1)	92.22	97.93	99.08	96.70	97.25	97.59	93.97	93.97	99.25	100.00	100.00												
13	Fadv_D_isolate_HBQ12_(KM096545.1)	92.22	97.93	99.08	96.70	97.25	97.59	93.97	93.97	99.25	100.00	100.00	100.00											
14	Fadv_IR/H1602.2/16(KY019221)	92.04	97.93	100.00	95.79	97.07	96.85	93.97	93.97	99.25	99.08	99.08	99.08	99.08										
15	Fadv_IR/H1603.1/16(KY019222)	91.85	97.74	99.82	95.60	96.89	96.67	93.79	93.79	99.06	98.90	98.90	98.90	98.90	99.82									
16	Fadv_D_strain_Indovax_FADV_mhr_17(MN537891.1)	90.48	97.14	99.08	93.91	96.85	94.56	93.15	93.15	99.64	99.27	99.27	99.27	99.27	99.08	98.90								
17	Fadv_D_isolate_FAdV-SAC115_(MK995484.1)	90.48	97.14	99.08	93.91	96.85	94.56	93.15	93.15	99.64	99.27	99.27	99.27	99.27	99.08	98.90	100.00							
18	Fadv_D_isolate_Pantnagar/HA-16/R-45_(MH379252.1)	90.48	97.14	99.08	93.91	96.85	94.56	93.15	93.15	99.64	99.27	99.27	99.27	99.27	99.08	98.90	100.00	99.67						
19	Fadv_E_isolate_IR/H651.3/13	66.48	65.22	66.85	64.83	65.19	64.80	67.42	67.42	66.10	66.30	66.30	66.30	66.30	66.85	66.67	66.30	66.30	66.30					
20	Fadv_E_isolate_IR/H651/13	66.48	65.22	66.85	64.83	65.19	64.80	67.42	67.42	66.10	66.30	66.30	66.30	66.30	66.85	66.67	66.30	66.30	66.30	100.00				
21	Fadv_IR/H651.3/13(KY019206)	66.48	65.22	66.85	64.83	65.19	64.80	67.42	67.42	66.10	66.30	66.30	66.30	66.30	66.85	66.67	66.30	66.30	66.30	100.00	100.00			
22	Fadv_E_isolate_HLJ/151129(KX077988.1)	66.23	65.34	66.97	64.94	65.31	64.93	67.17	67.17	66.22	66.42	66.42	66.42	66.42	66.97	66.79	66.42	66.42	66.42	97.22	97.22	97.22		
23	Fadv_IR/H651/13(KY019204)	66.48	65.22	66.85	64.83	65.19	64.80	67.42	67.42	66.10	66.30	66.30	66.30	66.30	66.85	66.67	66.30	66.30	66.30	100.00	100.00	100.00	97.22	
24	Fadv_A_(NC_001720.1)	59.70	61.19	62.76	61.08	61.08	61.02	60.92	60.92	61.88	62.57	62.57	62.57	62.57	62.76	62.94	62.38	62.38	62.38	58.05	58.05	58.05	59.47	58.05

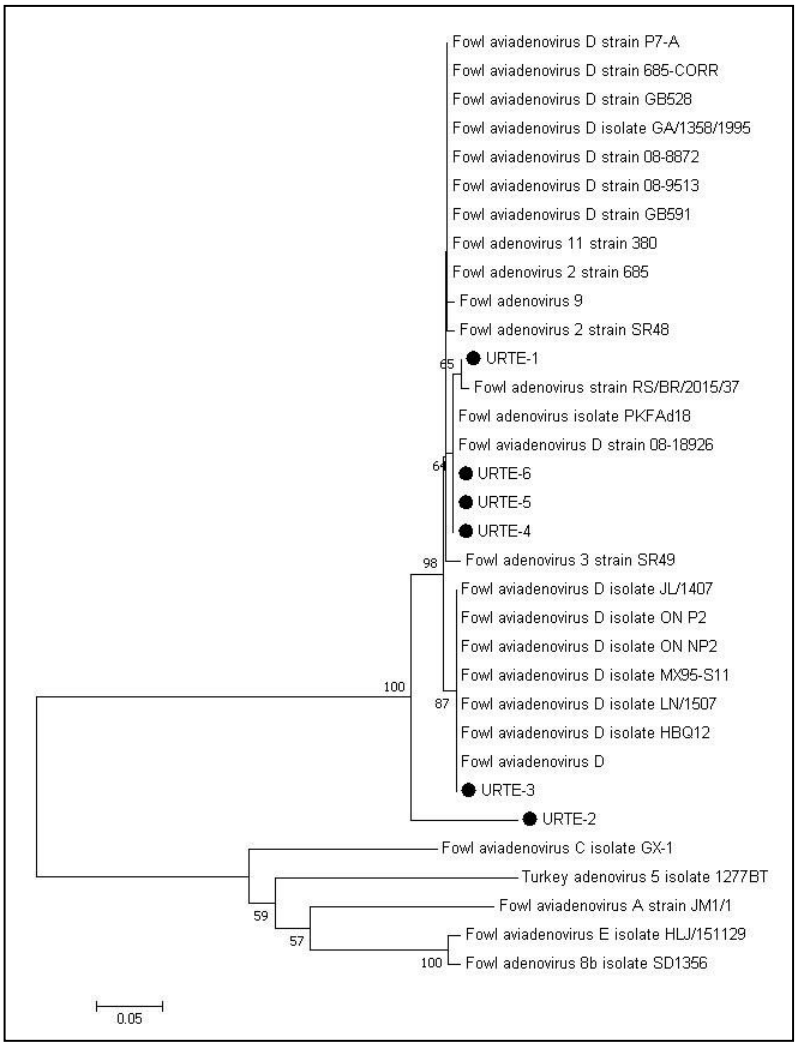

Figure 3. Phylogenetic tree of the 1406 bp region of the penton gene by MEGA7 software. Fowl adenoviruses detected in this study are shown with green squares, previously published Iranian strains are shown with black circles

Table 2. Sequence identity matrix for FAdV-D viruses detected in this study with other related fowl adenoviruses, based on the penton gene

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	URTE-1													
2	URTE-2	88.46												
3	URTE-3	97.80	88.46											
4	URTE-4	99.45	89.01	98.35										
5	URTE-5	99.45	89.01	98.35	100.00									
6	URTE-6	99.45	89.01	98.35	100.00	100.00								
7	Fowl_adenovirus_isolate_PKFAd18	99.45	89.01	98.35	100.00	100.00	100.00							
8	Fowl_aviadenovirus_D_strain_08-18926	99.45	89.01	98.35	100.00	100.00	100.00	####						
9	Fowl_adenovirus_strain_RS/BR/2015/37	99.45	87.91	97.25	98.90	98.90	98.90	98.90	98.90					
10	Fowl_aviadenovirus_D_isolate_GA/1358/1995	98.90	89.56	98.90	99.45	99.45	99.45	99.45	99.45	98.35				
11	Fowl_aviadenovirus_D_strain_GB528	98.90	89.56	98.90	99.45	99.45	99.45	99.45	99.45	98.35	100.00			
12	Fowl_aviadenovirus_D_strain_685-CORR	98.90	89.56	98.90	99.45	99.45	99.45	99.45	99.45	98.35	100.00	####		
13	Fowl_aviadenovirus_D_isolate_JL/1407	97.80	88.46	100.00	98.35	98.35	98.35	98.35	98.35	97.25	98.90	98.90	98.90	
14	Fowl_aviadenovirus_D_isolate_ON_NP2	97.80	88.46	100.00	98.35	98.35	98.35	98.35	98.35	97.25	98.90	98.90	98.90	100.00

4. Discussion

The IBH was primarily defined in the USA in 1963 (6). Subsequently, the disease spread throughout the globe, and the potency of the etiological agent to transmit both vertically and horizontally turned the disease into a complex problem (15). Apart from the fact that parents can transfer the virus to their chicks, the latent nature of the virus may pertain to making it unnoticeable for at least one generation (16). This might clarify the isolation of FAdVs from apparently healthy birds (17). Such convoluted nature and devastating effects associated with IBH infection necessitate investigations to prove the presence or absence of the virus in poultry farms.

There have been increased mortality rates due to IBH outbreaks in recent years in Iran. In 2021, mortality rates increased among 8-10-day-old broilers and hepatitis was an outstanding finding when they were necropsied. This research documented the presence of serotype 11 of FAdV in the North West of Iran. The first molecular detection was performed in 2012 in two broiler breeder flocks, in which FAdV serotype 11 was identified (18). In another investigation conducted on samples collected from 2013 to 2016 from broilers and broiler breeders, serotypes 8b and 11 of FAdV were found to be responsible (19). From 2017 to 2018, species D and E were diagnosed in broiler farms in the north of Iran with a morbidity rate of 70% (20).

This study provided an update on circulating FAdVs in Iran, and also introduced the penton gene as an alternative target for IBH diagnosis. The results revealed the presence of FAdV serotype 11 (species D) in broilers which is consistent with those of the previous studies. According to the phylogenetic results obtained from L1-hexon gene analysis, the previously reported FAdV-11 viruses in 2013 and 2016 are still circulating in the country, which may be due to the lack of any vaccination program against fowl adenoviruses (19). Apart from that, our strains shared high-level homologies with those identified in 1998 in Germany, 2005 in Canada, 2012 in China, 2017 in India, 2017 in Saudi Arabia, and 2018 in Pakistan.

Considering trade relations between Iran and Europe, and more recently, Iran and China, one hypothesis is that the virus has entered Iran from other countries via imported eggs and chicks. The relationship of the studied viruses with recent Pakistani and Arabian isolates is due to the strong relations of Iran with its neighboring countries. This viral transmission trade has been observed regarding many viruses, such as the foot-and-mouth disease virus, which usually moves from east to west, starting from India, passing through Afghanistan, Pakistan, and reaching Iran; consequently, it is spread in the Middle East (21).

The detection rate provided by the PCR targeting the penton gene was identical to the PCR targeting the partial hexon gene, and all the detected viruses were placed in the FAdV-D cluster in the penton-based phylogenetic tree. The sequence relationships of our strains with other related FAdVs were to a great extent

in agreement with those obtained from L1-hexon gene analysis.

Given that IBH is most frequently a primary disease in Iran (19) and has both horizontal and vertical routes of transmission, urgent preventive measures are needed. It is obvious that IBH has recently increased in broilers which necessitates the application of vaccines against this disease, especially in breeders from whom the viruses can be vertically transmitted.

Authors' Contribution

Study concept and design: GL-A and A-F.
Acquisition of data: A-F.
Analysis and interpretation of data: A-F, A-M, and T-A.
Drafting of the manuscript: A-F.
Critical revision of the manuscript for important intellectual content: A-M, GL-A, and T-A.
Administrative, technical, and material support: A-M, GL-A, and T-A.
Study supervision: A-M and GL-A.

Ethics

All experimental procedures and chicken handlings were carried out according to the standard animal experimentation protocols of the Veterinary Ethics Committee of the Faculty of Veterinary Medicine, Urmia University, West Azerbaijan, Iran (IR-UU-AEC/ 2380/PD/3).

Conflict of Interest

The authors declare that they have no conflict of interest.

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This study

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