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Original Article

Molecular identification of aviadenoviruses in broiler chickens suspected to inclusion body hepatitis in Golestan province, Iran

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Abstract

Background: Fowl adenoviruses (FAdVs) are distributed widely throughout the world, and domestic avian species of all ages are susceptible. Fowl aviadenoviruses (FAdVs) can be separated into 5 different species (A-E) with various genotypes and 12 serotypes. Some geno- or serotypes induce hepatitis-hydropericardium syndrome (HPS), inclusion body hepatitis (IBH), and adenoviral gizzard erosion (AGE). **Aims:** Detect FAdVs serologically and molecularly and sequencing of FAdVs in broiler flocks in Golestan province. **Methods:** From December 2017 to June 2018 liver tissues and blood samples were collected from 31 broiler flocks suspected of IBH. Polymerase chain reaction (PCR) was applied on liver samples and the positive samples were sequenced and antibody against FAdVs was measured by enzyme-linked immunosorbent assay (ELISA). **Results:** Out of 31 flocks, the titers of 29 flocks (93.5%) were high in ELISA test for FAdVs and 22 flocks (70.96%) were positive in PCR test. Sequence analysis indicated the isolates belonged to D and E genotype of adenovirus. **Conclusion:** Inclusion body hepatitis caused by FAdVs, are spreading increasingly in broiler flocks of Golestan province and more attention and surveillance programs of breeder and broiler farms are needed to develop preventive measures. Moreover, vaccination of poultry farms in Iran should be considered by more complement studies.

Key words: Adenovirus, Inclusion body hepatitis, Iran, Molecular identification, Serology

Introduction

The role of most aviadenoviruses as pathogens was not well-defined in the past but the recent data strengthened the role of some fowl aviadenoviruses (FAdVs) strains as primary pathogens, mainly in chickens. This could be demonstrated for some FAdV-1 strains (genotype A) causing gizzard erosion (GE) and some FAdV-4 strains (genotype C) which play major roles in the etiology of hydropericardium syndrome (HPS). In addition, other strains, mainly those belonging to genotype D and E, can cause severe liver damage leading to the condition known as inclusion body hepatitis (IBH) (Faldy *et al*., 1976; Marek *et al*., 2010; Mettifogo *et al*., 2014; Hess, 2020). Adenoviruses can be transmitted vertically from eggs. Horizontal transmission is also possible by contact with faeces. Airborne transmission is not feasible unless birds have previously been exposed to pathogens that cause respiratory impairment. The disease is common in broiler chickens and usually starts at 7 days of age. The causative agents are capable of creating intranuclear eosinophilic inclusion bodies in the liver. Gross lesions are anemia, jaundice in the skin and subcutaneous fat, hemorrhages in various organs especially muscles, and degeneration of the bone marrow (McFerran and Adair, 1977). High mortality rates in challenge with acute FAdV-4 strains and growth retardation in IBH infection and GE have caused huge economic losses (Ono *et al*., 2003; Balamurugan and Kataria, 2004). In broiler chickens, the highest rate of virus shedding is between 4 and 6 weeks of age. Due to cross immunity, it is usually not possible to isolate different serotypes from the same bird and neutralizing antibodies for a serotype prevents the proliferation of other serotypes. At the peak of egg production, FAdV is often diagnosed probably because of the stress of egg production or the increase in sex hormones and, as the result, virus re-activation and increasing vertical transmission (Hess, 2020). Based on the reports of broiler chickens suspected of IBH in Golestan province in Iran, this study was conducted to detect avian adenoviruses serologically and molecularly in the area.

Materials and Methods

Sampling

Samples were collected from 31 broiler flocks suspected of IBH in different parts of Golestan province (longitude 55.047917 and latitude 37.155483, raising 9

million broiler chicks every 42 days) in Iran. All of the samples were collected from farms suspected of IBH showing the clinical signs of depression, respiratory and digestive signs and mortality between 10 and 30%. At necropsy, enlarged and pale yellow livers with multiple petechial hemorrhages were noticed and 10 liver samples per flock were collected. At the same time blood samples from wing vein of 10 birds with clinical signs were collected and along with dried ice were sent to the laboratory. In the laboratory, the liver samples were frozen at -70°C while the sera were stored in the microtubes at -20°C upto examination.

Serological test

Antibody levels against IBH were measured using Biocheck FAdV enzyme-linked immunosorbent assay (ELISA) kit (Biocheck, Product Code: CK132, Netherlands) according to the ELISA kit instructions.

Molecular detection

DNA extraction

DNA of liver tissue specimens was extracted by Sinaclon DNA Extraction kit (Sinaclon Co., Iran) and according to the manufacturer's instructions. For this purpose, 50-100 mg of each tissue sample was divided into small pieces using a sterile scalpel and placed inside 2 ml microtubes. For homogenization, 100 µL of prelysis buffer and 20 µL of ributinase, were added to the microtube and stored at 55°C for 3 h in laboratory water bath. During this time, microtubes were repeatedly vortexed in 5 min intervals, each time for 5 s with a Vortex mixing machine. All DNA extraction steps were performed carefully according to the manufacturer's instructions. Finally, the extracted DNA was stored at -80°C in 1.5 ml microtubes.

Polymerase chain reaction (PCR)

The 590 bp region of the partial *Hexon* gene (NCBI accession number: MG738474) was amplified using a pair of specific primers (Table 1) to confirm the presence of viral DNA (Nateghi *et al*., 2014). All positive PCR products were sequenced by BioNeer Co., South Korea.

Table 1: Primers used to detect aviadenoviruses

Primer name	Primer sequence
Hex L1-F	5´-ATGGGAGCSACCTAYTTCGACAT-3´
Hex L1-R	5´-AAATTGTCCCKRAANCCGATCTA-3´

The PCR was performed in a 20 µL reaction volume containing 10 µL of 2x Ampliqon PCR master mix with 1.5 mM $MgCl_2$, 0.5 µL of each primer (10 Pmol/µL), 6 µL of ddH_2O and 3 µL extracted viral DNA. The thermocycler (Quanta Biotech, Germany) was configured as 94°C for 5 min followed by 35 cycles of 94°C for 60 s, 54.2°C for 45 s, 72°C for 60 s, and a final step at 72°C for 5 min. Negative (ddH_2O instead of DNA) and positive (the DNA from Australian FAdV-8b vaccine strain (Intervet Pty Ltd., Australia) controls were also added to confirm the accuracy of the PCR results. The PCR products (590 bp) were then electrophorized using 1% agarose gel (Fig. 1). All of the reverse transcription-polymerase chain reaction (RT-PCR) products were cut from the gel, purified by the AccuPrep PCR Purification kit (BioNeer Co., South Korea) according to the manufacturer's protocol. Finally, all of the PCR products were sequenced by BioNeer Co., South Korea. The grouping and typing were supported by phylogenetic analysis (Fig. 2).

Results

The serology tests indicated that 29 farms were positive. The minimum titer was 100 (negative) and the maximum titer was 9166 (positive). In the molecular detection of the liver samples, 22 out of 31 flocks were positive by both PCR and serological tests. It is important to mention that while 9 flocks were negative by PCR test, only two flocks were negative in both ELISA and PCR, which means 7 flocks were PCR-negative but serologically positive (Table 2).

The sequence of 16 positive PCR products out of 22 positive samples was determined by Bioneer (Bioneer Co., South Korea). A phylogenetic tree (Fig. 2) was

Table 2: Molecular and serological test results

Flock No.	ELISA			PCR result
	Result	Mean titer	CV%	
1	Neg	100	75	Neg
2	Neg	235	244	Neg
3	Pos	3691	106	Pos
4	Pos	5767	54	Neg
5	Pos	8878	2	Pos
6	Pos	8820	3	Pos
7	Pos	8685	3	Pos
8	Pos	8601	3	Pos
9	Pos	8537	2	Pos
10	Pos	9015	9	Pos
11	Pos	9151	2	Neg
12	Pos	7298	29	Neg
13	Pos	9098	3	Neg
14	Pos	9166	6	Neg
15	Pos	8784	9	Neg
16	Pos	9019	5	Pos
17	Pos	6271	32	Pos
18	Pos	8988	2	Pos
19	Pos	8897	2	Pos
20	Pos	8659	2	Pos
21	Pos	8612	2	Pos
22	Pos	8584	2	Pos
23	Pos	8833	4	Pos
24	Pos	8900	2	Pos
25	Pos	8807	3	Pos
26	Pos	4117	67	Pos
27	Pos	7647	27	Pos
28	Pos	8896	3	Pos
29	Pos	8344	13	Pos
30	Pos	7791	30	Pos
31	Pos	8526	8	Neg

CV: Coefficient of variation, PCR: Polymerase chain reaction, ELISA: Enzyme-linked immunosorbent assay, Pos: Positive, and Neg: Negative

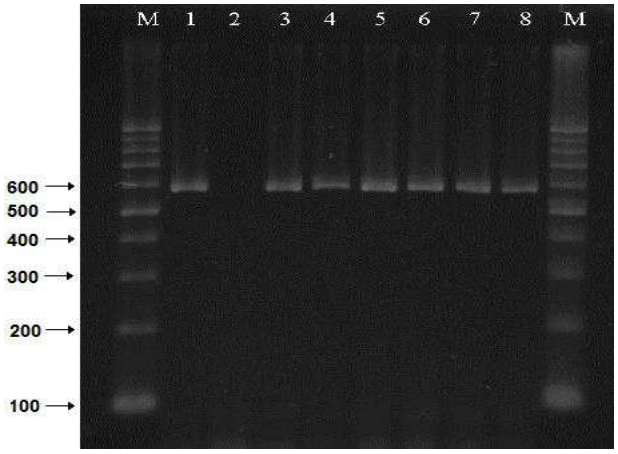

Fig. 1: Amplification of *Hexon* gene of FAdV. Lane M: Marker 100 bp, Lane 1: Positive control (590 bp), Lane 2: Negative control, and Lanes 3-8: Positive samples

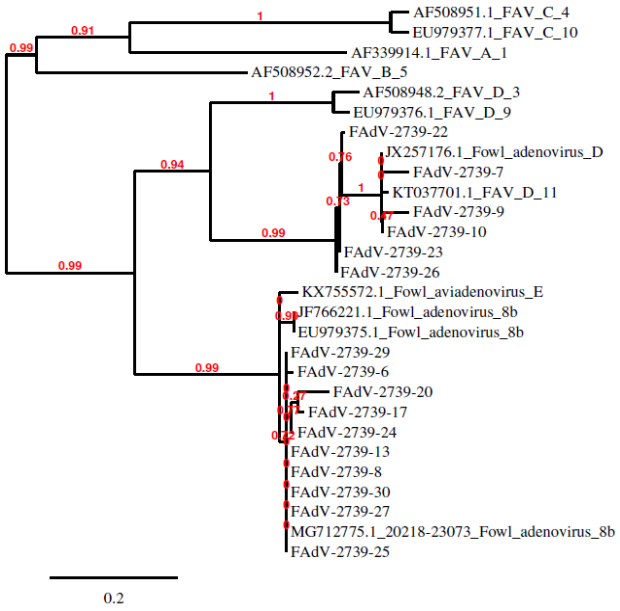

Fig. 2: The phylogenetic tree of the L1 loop of the *Hexon* gene based on the *Hexon* gene sequence of FAdVs isolates

Table 3: GenBank accession numbers for 16 Golestan, Iran fowl adenovirus FAdVs isolates

Isolate	Accession number	Genotype
FAdV-2739-6	MK850433	E (8b)
FAdV-2739-8	MK850434	E (8b)
FAdV-2739-13	MK850435	E (8b)
FAdV-2739-17	MK850436	E (8b)
FAdV-2739-20	MK850437	E (8b)
FAdV-2739-24	MK850438	E (8b)
FAdV-2739-25	MK850439	E (8b)
FAdV-2739-27	MK850440	E (8b)
FAdV-2739-29	MK850441	E (8b)
FAdV-2739-30	MK850442	E (8b)
FAdV-2739-7	MK867683	D
FAdV-2739-9	MK867684	D
FAdV-2739-10	MK867685	D
FAdV-2739-22	MK867686	D
FAdV-2739-23	MK867687	D
FAdV-2739-26	MK867688	D

constructed based on the nucleotide sequences of the L1 *Hexon* gene and the corresponding regions of the other avian adenovirus (AAV) strains rescued from GenBank. The nucleotide sequences of the *Hexon* gene were also compared to the AAV sequence data available in the National Center for Biotechnology Information database (http://ncbi.nlm.nih. gov) using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the phylogenetic relationship was established by http://www.phylogeny.fr/simple_phylogeny.cgi. Phylogenetic analysis of *Hexon* gene sequences showed that isolates from six and ten farms belonged to genotype D/serotype 11 and genotype E/serotype 8b of aviadenoviruses, respectively. These isolates shared 98%-99% homology with the previously identified isolates from Iran, China, Canada, and Australia. The sequences of *Hexon* genes isolated in this study have been saved in GenBank with accession numbers indicated in Table 3.

Discussion

Currently, IBH/HPS is considered as an economically important disease in several countries. Aviadenovirus infections can have no signs or present with a variety of signs (Nateghi *et al*., 2014). The importance of the presence of adenovirus infections is not only due to economic losses associated with the mortality (10-30%) and reduced performance, but also due to the effects of the virus in interacting with other viruses (Hafez, 2011). The first tissue affected by IBH is the liver. The liver is pale, friable and swollen with some possible small white patches and petechial hemorrhages (Grafl *et al*., 2012). The gross lesions of livers reported in the studies were similar to other researchers such as Hosseini and Morshed (2012), Nateghi *et al*. (2014), and Tafti *et al*. (2016). Some studies about the prevalence of adenovirusis in Iran over recent years have been carried out in some provinces, including Mashhad, Isfahan, Qom, and Khuzestan (Nateghi *et al*., 2014; Tabib Ghafari *et al*., 2017). In this study, all samples were collected from the flocks with clinical signs and gross lesions suspected of IBH in Golestan province from January to July 2018, and the high percentage of positive samples serologically (29 flocks, 93.5%) and molecularly (22 flocks, 70.96%) indicated high infection rate of broiler flocks with IBH, which seems to cause economically significant losses to the Golestan poultry industry. There are also seven flocks with high titers in (FAdVs) ELISA test but in molecular detection they were negative, which could be due to the virus elimination over post infection time, or maybe PCR not being able to detect the virus.

There was no comprehensive study available regarding how long the virus is detectable in the liver tissue after the challenge/infection.In recent years in Iran, some limited studies have been performed to detect adenoviruses molecularly in some provinces including Mashhad, Isfahan, Qom, and Khuzestan (Nateghi *et al*., 2014; Tabib Ghafari *et al*., 2017). The isolates detected

in this study were FAdV-D serotype 11 and FAdVs-E serotype 8b. The most common problems associated with the avian adenovirus, IBH, are caused by serotypes 2, 3, 6, 7, 8, 9, and 11 in Australia and New Zealand (Cowen, 1988), in Canada, by serotypes 2, 8, and 11 (Hafez, 2011). Several different serotypes can cause IBH infection in birds, and in the last 10 years, the most reported IBH-induced serotypes belonged to D and E genotypes (Steer *et al*., 2011). In the study of Zadravec *et al*. (2011), IBH was induced by serotype 8 and the adenoviruses isolated by De Herdt *et al*. (2013) belonged to the serotypes 1, 2, 3, 5, 8, and 11. Nateghi *et al*. (2014) reported that adenovirus isolates belonged to serotypes 8b, 2 and 11 (D and E genotypes). Also, Morshed *et al*. (2017) identified 24 FAdVs in broiler and broiler breeder flocks in seven different provinces of Iran, all of which belonged to genotype D (serotype 11) and E (serotype 8b). These studies suggested species D and E as the dominant species in Iran, which is in agreement with the results of this study in Golestsn province. The studies in Canada supported the hypothesis that IBH in broilers can occur as a primary disease with no known immunosuppressive involvement (Gomis *et al*., 2006). It is a widespread belief that FAdVs are ubiquitous in chicken populations, independent of the health status of the birds. All samples in this study were collected from poultry farms suspected to IBH based on the clinical signs and gross lesions and also ELISA test was performed in parallel with PCR to confirm the IBH. Since no vaccine is licensed for FAdVs in Iran, and the presence of IBH has been proved in our study in broiler flocks in Golestsn province and previous studies in broiler flocks and broiler breeder flocks of Iran also confirmed this (Hosseini and Morshed, 2012; Morshed *et al*., 2017). It seems IBH-induced adenoviruses are spreading increasingly in broiler flocks due to vertical and horizontal transmission which can cause significant loss in the poultry industry. Moreover, in present study, seven flocks were found to have high titers of FAdVs in ELISA test, but adenovirus in their liver tissue was not detected by PCR test, which indicates that the virus was eliminated over time, or the PCR test was not able to detect the virus over time post infection. In conclusion, IBH-induced adenoviruses are spreading increasingly in broiler flocks in Golestan province and need more attention and surveillance programs of breeder and broiler farms to develop preventive measures and also vaccination of poultry farms in Iran should be considered by more complement studies.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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