

Short Paper

Molecular characterization of avian adenoviruses in Iranian broiler flocks

Nateghi, E.¹; Razmyar, J.^{2*} and Bassami, M. R.^{2,3}

¹Graduated from Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; ²Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; ³Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

*Correspondence: J. Razmyar, Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: jrazmyar@um.ac.ir

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Summary

This study was conducted to molecularly detect avian adenoviruses in broiler flocks showing liver lesions and respiratory syndrome in northeast Iran. In total, 60 tissue samples were collected from broiler farms with liver lesions, respiratory syndrome and also from clinically healthy flocks. Six samples were positive; however, three samples were selected for molecular studies. PCR products were sequenced to confirm the identity of avian adenoviruses. Based on the sequence analysis of the L1 region of the hexon gene, the NRB/FAV/4 should be classified as FAdV 8b strain and two other isolates - NRB/FAV/1 and NRB/FAV/5 - classified in cluster of the FAdV 2 & 11. As far as we know, this preliminary investigation is the only documented study to confirm the presence of avian adenoviruses in broiler flocks in Iran.

Key words: Avian adenoviruses, Broiler, Inclusion body hepatitis (IBH), Hexon gene, PCR

Introduction

Viruses belonging to Adenoviridae family (AdVs) are common infectious agents in humans, poultry, and wildlife worldwide (Fitzgerald, 2008). Adenoviruses are classified into four genera: Mastadenovirus (from mammalian hosts) (Benko *et al.*, 2000), Aviadenovirus (from avian hosts) (Harrach *et al.*, 1997), and the two genera Atadenovirus and Siadenovirus. Atadenoviruses have been found in birds (Harrach *et al.*, 1997; Hess *et al.*, 1997), mammals (Thomson *et al.*, 2002), and reptiles (Benko *et al.*, 2002). Siadenoviruses have been found in birds (Pitcovski *et al.*, 1998) and amphibians (Davison *et al.*, 2000).

Inclusion body hepatitis (IBH) is an emerging, economically important disease of 3–6-week-old broiler chickens (Balamurugan and Kataria, 2004). Historically, IBH was generally considered a secondary disease in broiler associated with primary immunosuppression following infection with IBDV or CIAV (Gomis *et al.*, 2006). According to the American classification, 12 fowl serotypes (FAdV1 to FAdV12) have been recognized till now (McFerran, 1997). These 12 FAdV serotypes have been classified in five species (fowl adenovirus A to E) (Benko *et al.*, 2000).

The aim of the present study was detection of AAVs in Iranian broiler flock.

Materials and Methods

Samples

Cases of liver lesions, respiratory syndrome with

purulent air sacs and lungs and clinically normal found at necropsy or clinical examination were collected from 60 broiler flocks referred to the Faculty's Poultry Clinic from different parts of the northeast of Iran. The lung and livers of each flock (1-5 samples) were pooled after being processed as described below and finally 60 samples were chosen and included in this study. In the laboratory, the samples were frozen or placed in tubes containing TNE buffer (1 mM EDTA, 100 mM NaCl, 10 mM TrisHCl, pH = 8.0). Each tissue sample was divided in two halves. One half was cut into fine pieces and the volume increased 5 times by TNE buffer (pH = 8.0). Remaining half was stored at -70°C. The tissue homogenates were prepared as described previously. Briefly, fine pieces of tissue's sample in TNE buffer were vortexed vigorously, frozen and thawed 3 times, and centrifuged at 14000 × g in 4°C for 20 min. The supernatant was removed, transferred to new 1.5 ml RNase and DNase free microtubes, and stored at -70°C until further use.

DNA extraction and polymerase chain reaction

To amplify 590 bp target product, viral DNAs were extracted by DNA extraction kit (BIONEER®, South Korea). The procedures were carried out according to the manufacturer's instructions. PCR was carried out by using published primers Hex L1-s (5'- ATGGGAGCSA CCTAYTTCGACAT-3') and Hex L1-as (5'- AAATTG TCCCKRAANCCGATGTA-3') that corresponded to nucleotides 301-323 and 868-890 of the hexon gene, respectively and included the sequence encoding the L1 loop of the hexon gene (Raue *et al.*, 2005). The

amplification was carried out in 25 µl reaction volume consisting of 2.5 µl 10 x PCR buffer, 1 µl 10 mM dNTPs, 1.5 µl of each primer (10 pmol/µl), 0.5 µl Taq DNA polymerase (5 U/µl), 1 µl 50 mM MgCl₂, 12 µl of ddH₂O, and 5 µl total DNA extract and was programmed in a thermocycler (Techno Mastercycler, England) as follows: 95°C for 3 min followed by 37 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 5 min. In all PCR reaction sets, negative controls (ddH₂O instead of DNA) and positive control were included. The initial suspected isolated one was subjected to DNA extraction and PCR amplification followed by DNA sequencing, and considered as positive control. The amplification products were detected by gel electrophoresis. Gels were run for 1.5 h at 80 V, stained with ethidium bromide (0.5 µg/ml), exposed to ultraviolet light and photographed.

Sequencing and phylogenetic analysis

Three PCR products (590 bp), including respiratory and liver lesion's isolates, were submitted for automated sequencing in both directions at the BIONEER[®] (South Korea). PCR primers as sequencing primers have been used. Nucleotide and predicted amino acid sequence data were aligned with the clustal alignment algorithm. Phylogenetic analysis based on nucleotide sequences was conducted using a distance method, unweighted pair group with arithmetic mean, by calculating bootstrap values for 1000 replicates in CLC Main Workbench Package Version 5 (CLC Bio, Aarhus, Denmark). The FAdV sequences used for comparative analysis include FAV1 (ACL68135), FAV2 (ACL68136), FAV3

(ACL68137), FAV4 (ACL68138), FAV5 (ACL68139), FAV6 (ACL68140), FAV7 (ACL68141), FAV8a (ACL68142), FAV8b (ACL68143), FAV9 (ACL68144), FAV10 (ACL68145), and FAV11 (ACL68146).

Results

To the best of our knowledge, this is the first report of fowl adenovirus's outbreaks in Iranian poultry flocks. Six out of 60 tissue samples (10%) were positive by PCR in this study. 6 out of 40 (15%) suspected samples were as follow:

Five samples out of 20 inflamed and enlarged liver tissue samples (25%)

One out of 20 congested and involved lung tissue samples (5%)

None of the twenty healthy livers were positive (Table 1)

The sequence analysis of PCR products has been exactly aligned and 90 to 100% identical to reference sequences of two main subgroups of Aviadenoviruses deposited in GenBank. The first positive PCR product was subjected to sequencing and after blasting in GenBank to confirm being a positive reaction belonging to FAdVs, was used as positive control in further reactions. Based on sequence analysis of the L1 region of the hexon gene, the NRB/FAV/4 should be classified as FAdV 8b strain and two other isolates: NRB/FAV/1 and NRB/FAV/5 classified in cluster of the FAdV 2 & 11.

Discussion

FAdVs related diseases such as HPS, Gizzard

Table 1: Summary of positive field cases included in the study

Flock No.	Isolate	Flock size	Age (d)	Category*	Sequenced	Mortality %	Serotype
1	NRB/FAV/1	10000	15	II	Done	6	FAD 11/2
4	NRB/FAV/4	15000	18	II	Done	12	FAD 8b
5	NRB/FAV/5	20000	34	I	Done	23	FAD 11/2
7	NRB/FAV/8	20000	19	II	ND	7	ND
9	NRB/FAV/11	10000	27	II	ND	9	ND
23	NRB/FAV/12	20000	23	II	ND	6	ND

* I: Respiratory syndrome flocks, II: Liver lesion's flocks, and ND: Not done

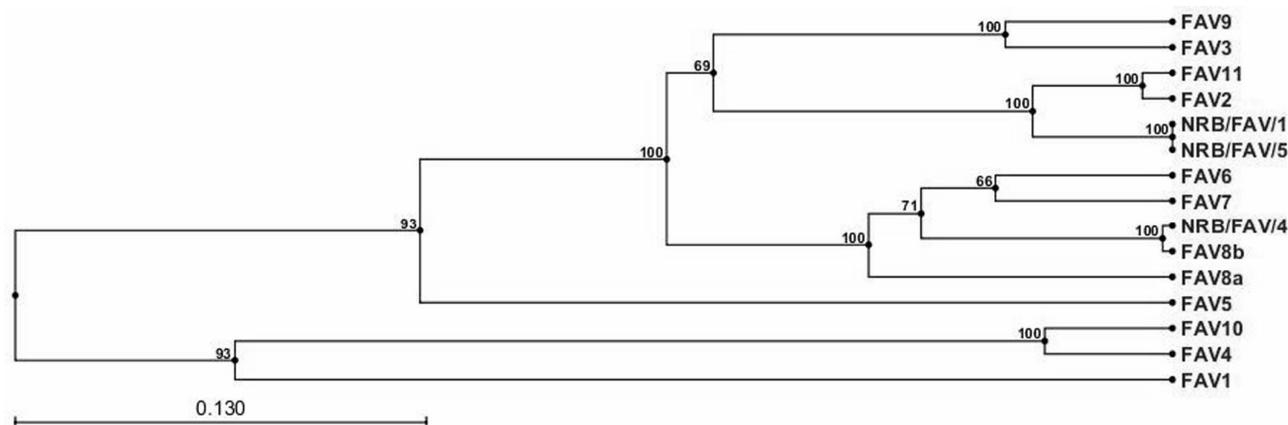


Fig. 1: Phylogenetic tree of nucleotide sequence from the 590 bp L1 hexon gene of Iranian AAVs field isolates and published serotypes. The tree was constructed by the clustal method by calculating bootstrap values for 1000 replicates. Branched distances correspond to sequence divergence

erosions and IBH are emerging, economically important diseases which are known in some countries. Avian adenovirus infections can be without symptoms or with a variety of symptoms (Shamim *et al.*, 2009). The sensitivity of virus isolation is lower than PCR methods within tissue samples. Virus isolation requires more than one passage for CPE appearance in cell cultures, which takes about three weeks approximately (Cowen *et al.*, 1996). The FADVs group-specific PCR has been noted to be a rapid, sensitive and specific test to demonstrate all different serotypes of avian adenovirus infection and might be used for avian adenovirus surveillance in many countries, routinely (Xie *et al.*, 1999). It is well known that approximately 15% of the entire FAdV genome represent serotype-specific epitopes, and that the classification of serotypes by analysis of the nucleotide sequence of the amplified L1 loop of hexon protein is an efficient method (Ojkic *et al.*, 2008). In the present investigation, PCR with the primers Hex L1-s and Hex L1-as was used for serotype identification of six isolates as it has proven for the identification of FAV serotypes (Raue *et al.*, 2005). In an interesting similarity with our findings based on a research in Canada, out of 287 samples that were further analysed by hexon gene loop 1 sequencing analysis, FADVs associated with IBH outbreaks were genetically related to FAdV 2 (9 isolates, 99.4% identity), FAdV 8a (100 isolates, 99.4 to 100% identity) and FAdV 11 (98 isolates, 99.4 to 100% identity) (Benko *et al.*, 2005). In this study, phylogenetic analysis based on nucleotide sequences divided the sequenced Iranian FADVs into two groups, branch A consists of FAV 8b and NRB/FAV/4 with 99.38% identity and a separate branch included NRB/FAV/1 and NRB/FAV/5 that showed closer relation to FAdV 2 & 11 with 90.62 and 92.50%, respectively. FADVs are known to be causative agents of respiratory tract infection in chickens; in one report, out of 13 respiratory disease outbreaks, adenoviruses were isolated from all 13 outbreaks (Dhillon *et al.*, 1982; Dhillon and Kibenge, 1987). In our study, we only found one out of 20 samples with respiratory lesions, positive in PCR assay. In a research from registered isolates, clinical and necropsy findings during 20 years including hundreds of adenovirus isolates, showed no primary role for adenovirus in poultry respiratory disease (Adair and Fitzgerald, 2008). Adenoviruses cause lesions accompanied with the other respiratory viruses and may be in countries where the other respiratory factors are higher and there is no control program, detection of adenovirus has a higher chance. To evaluate the current status of FADV infections in Iranian poultry flocks, it seems epidemiologic studies that include extensive serological surveys are necessary when making a decision for using vaccination programs to reduce economic losses caused by FADV infections.

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