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Fowl Adenoviruses D and E Cause Inclusion Body Hepatitis Outbreaks in Broiler and Broiler Breeder Pullet Flocks

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SUMMARY. Twenty-four fowl adenoviruses (FAdVs) were isolated from broiler and broiler breeder pullet flocks in Iran during 2013–2016 and were identified and characterized. All FAdVs were from inclusion body hepatitis (IBH) cases, showing an enlarged and pale yellow liver with multiple petechial hemorrhages. Phylogenetic analyses of partial hexon gene sequences are an adequate and quick method for differentiation and genotyping. The isolates were subjected to PCR to amplify a 590-bp fragment from the hexon gene. Sequence analysis revealed the presence of two species D and E. Eighty FAdV isolates were genetically related to the strain EU979378 of FAdV-11 (96.5% to 97.6% identity), and six isolates were related to the strain EU979375 of FAdV-8b (97% identity). The results indicated that two FAdV serotypes (11 and 8b) are high prevalence serotypes of FAdVs in Iran and are pathogenic enough to cause IBH in young chickens. Therefore, preventive measures against FAdV infection on poultry farms should be implemented.

RESUMEN. Adenovirus de pollo D y E causantes de brotes de hepatitis con cuerpos de inclusión en parvadas de pollos de engorde y reproductores. En el periodo comprendido entre 2013 y 2016 se aislan y caracterizaron veinticuatro adenovirus de pollo (FAdV) de parvadas de pollos de engorde y de pollas reproductoras pesadas en Iran. Todos los adenovirus se obtuvieron de casos de hepatitis con cuerpos de inclusión, que mostraron un hígado agrandado, amarillo pálido con múltiples hemorragias petequiales. Los análisis filogenéticos de secuencias parciales del gene del hexon son un método adecuado y rápido para la diferenciación y genotipificación. Los aislamientos se analizaron mediante PCR para amplificar un fragmento de 590 pares de bases del gene hexon. El análisis de secuencias reveló la presencia de dos especies, D y E. ochenta aislamientos de adenovirus estaban genéticamente relacionados con la cepa EU979378 de adenovirus de las gallinas serotipo11 (96.5% a 97.6% de identidad) y seis aislados estaban relacionados con la cepa EU979375 del adenovirus de pollo serotipo 8b (97 % de identidad). Los resultados indicaron que dos serotipos de adenovirus del pollo (11 y 8b) son los serotipos de alta prevalencia en Iran y son lo suficientemente patógenos como para causar hepatitis de inclusión en pollos jóvenes. Por lo tanto, se deben implementar medidas preventivas contra la infección por adenovirus de las gallinas en granjas avícolas.

Key words: fowl adenovirus, inclusion body hepatitis, hexon gene, genotyping, Iran

Abbreviations: AdVs = adenoviruses; CIAV = chicken infectious anemia virus; FAdV = fowl adenovirus; HHS = hydropericardium syndrome; IBDV = infectious bursal disease virus; IBH = inclusion body hepatitis

Adenoviruses (AdVs) are nonenveloped double-stranded DNA viruses, which belong to the family Adenoviridae. The family Adenoviridae is currently divided into five genera: Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus, and Ichtadenovirus. Chickens can be infected by fowl adenoviruses (FAdVs), belonging to the genus Aviadenovirus, egg drop syndrome virus (duck adenovirus A, genus Atadenovirus), and turkey hemorrhagic enteritis virus (turkey adenovirus A, genus Siadenovirus) (25).

FAdVs are transmitted vertically and horizontally via all excretions, but the highest titers are found in feces (5). FAdVs have been isolated from healthy and diseased domestic fowl, and the most important diseases associated with FAdVs in chicken are inclusion body hepatitis (IBH), hydropericardium syndrome (HHS), and gizzard erosion (10,16). The role of these viruses as primary pathogens is unclear (22,23).

FAdVs are classified into five different species (FAdV-A to FAdV-E) due to their molecular structure (6) and also into 12 serotypes (FAdV-1 to -8a and -8b to -11), as a result of cross-neutralization tests (6). Recently, at least 12 genotypes were identified within the five FAdV species based on the hexon gene sequences (9,19).

Most commonly, FAdVs isolated from IBH cases belong to FAdV-D and FAdV-E (6,17,20,24). FAdV strains related to HHS belong to FAdV-4 (FAdV-C), and they are highly pathogenic to chickens (7). FAdV-1 (FAdV-A) has been isolated from most cases of gizzard erosion (18). PCR using different primer sets, which can amplify the various regions of the hexon gene, followed by restriction enzyme digestion or nucleotide sequencing of the products, allows the differentiation of field isolates to species and serotypes (13). In addition, the combination of PCR and high-resolution melting curve analysis has provided an accurate and rapid genotyping technique for the identification of FAdV serotypes (19).

In recent years, the number of IBH cases has increased in different
provinces of Iran, particularly in broiler flocks (8,15). The current study describes the isolation and molecular typing of FAdV isolates from different regions of Iran between 2013 and 2016.

MATERIALS AND METHODS

Field and clinical data. The number of IBH cases has increased in Iranian chicken flocks in recent years. Chickens from broiler and broiler breeder pullet flocks with various clinical signs of IBH from different regions of Iran between 2013 and 2016 were submitted to the PCR Veterinary Diagnostic Laboratory (Tehran, Iran). The FAdVs were isolated from 24 different broiler and broiler breeder flocks. Adenovirus vaccines are not commercially available in Iran, so none of chicken flocks were vaccinated against adenoviruses.

Isolation. Liver samples were homogenized. The supernatant obtained after centrifugation of the homogenized tissues was filtered (0.2-μm filters) and inoculated into 5-to-7-day-old specific-pathogen-free chicken embryos via the yolk sac route (2).

PCR for amplification of hex loop 1 gene. Viral DNA was extracted from the chorioallantoic fluid and infected liver tissues, according to the previously described method (8). Amplification of a 500-bp region of the hexon gene was done by PCR to confirm the presence of viral DNA. One set of primer binding to hex loop 1 (L1) gene was used to amplify L1. The primers were forward primer: Hex L1-5'-ATGGGAGCSACCTAYTTCGACAT-3' and reverse primer: Hex L1-as 5'-AAATTGTCCCKRAANCCGATGTA-3' (19). A 25-μl reaction consisted of 4 μl of deoxynucleoside triphosphate at 1.25 mM, 2 μl of each primer at 25 μM, 5 μl of 5X PCR buffer, 1.25 U of DNA polymerase (SinaClon, Karaj, Iran), 2 μl of extracted viral DNA, and 2 μl of 25 mM MgCl2. PCR assays were performed in a Gradient Palm-Cycler (Corbett Life Science Pty, Ltd., Mortlake, Australia). The conditions of the PCR were a denaturation step of 94 C for 2 min, followed by 40 cycles, consisting of 94 C for 20 sec, 56 C for 20 sec, and 72 C for 30 sec, as described by Steer et al. (19).

Sequencing. PCR products were purified by using the High Pure PCR Product Purification Kit (Roche, Qiagen, Hilden, Germany). Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA) in forward and reverse directions. The nucleotide sequences of hexon genes were compared with the FAdV sequences data available in the National Center for Biotechnology Information, and the phylogenetic relationship was established. All of the sequences were aligned using the CLUSTALW with MEGAS5 software (The Biodesign Institute, Arizona, USA). Distance-based neighbor-joining trees were constructed by using the Tamura-Nei model (21). The robustness of the phylogenetic trees was assessed by 1000 bootstrap replicates. Bootstrap support of ≥80% was shown near the nodes in the phylogenetic trees.

Accession numbers. The sequences of the hexon protein gene were submitted to GenBank under the accession nos. KY019203–KY019226.

RESULTS

Twenty-two FAdV isolates from broilers and two FAdV isolates from broiler breeder pullets were typed by PCR and sequence analysis, as shown in Table 1. All isolates were obtained from diseased flocks with IBH clinical signs, such as inappetence, lethargy, huddling with ruffled feathers, and sudden onset of mortality. Sometimes, yellow mucoid droppings could be seen. FAdV infection occurred in broilers between the ages of 9 to 25 days old. Mortality ranged from negligible to 30% and peaked within 3 to 4 days and ceased within 2 wk. At necropsy, the livers were friable, enlarged,
Fig 1. FAdV phylogenetic tree of the hex loop 1 of the hexon gene by using the MEGA5 package. The tree is based on the sequence of the hexon gene from some selected Iranian FAdVs isolates, reference strains, and some FAdVs from other counties. The viruses characterized in this article are indicated by black diamonds and boldface.
and pale yellow-white with multiple petechial hemorrhages. Petechial or ecchymotic hemorrhages may be observed in the skeletal muscles. Kidneys were pale and swollen. Hepatitis sometimes led to jaundice. The crops were full, and birds usually died in good body condition.

According to the results of phylogenetic analysis, based on the partial hexon gene sequences (shown in Fig. 1), all of the isolates were located in FAdV-E serotype 8b and FAdV-D serotype 11. The most frequently identified types (75%) within these two species were species D serotype 11. Six isolates (25%) were grouped into species E serotype 8b. The species D viruses matched as 96.5% to 97.6% identical to FAdV-11 reference strain EU979378, and the viruses of species E matched as 97% identical to FAdV-8b reference strain EU979375. The identity ranges among Iranian FAdV-D serotype 11 are between 98.4% and 100% (Table 2). The FAdV-D serotype 11 can be divided to three clusters (Fig. 1). Eighteen FAdV-D isolates from Iran are placed in Clusters II and III. The identity within Iranian FAdV-D in Clusters II and III are ≥98.8%. The FAdV-D isolated from Canada (EF685644), China (KM0965451), India (KM250091), Korea (HQ697595), and the United States (DQ323984) are placed in Cluster III. Iranian FAdVs in Cluster II had 99.2% to 100% identity with FAdV from Brazil (FJ360747). In serotype 8b, the exact sequences of six strains were the same (100% identity) and had 100% identity with three strains from Italy, Austria, and Hungary (HM592279, FN869965, and KC750803; Fig 1). Six isolates had 96.1% identity with another FAdV strains serotype 8 from Iran (NRB/FAV/4 hexon) (15). Two broiler breeder isolates belonged to Cluster III, with the highest identity to strains from Canada, China, Korea, and the United States.

**DISCUSSION**

FAdVs are classified into five species (A to E), based upon their phylogenetic relationships, restriction enzyme fragmentation, pathogenicity, cross neutralization, and recombination potential (7). We analyzed 24 FAdV isolates recovered from different regions of Iran, based on nucleotide sequence diversity. The isolates originated from broilers and broiler breeder pullets, showing mortality and yellow livers with multiple petechial hemorrhages at necropsy. FAdV-E serotype 8b and FAdV-D serotype 11 were identified out of the 24 FAdV isolates. This finding suggests these serotypes are dominant in Iran and can cause natural clinical diseases in chickens. Previously, IBH in Iran has not attracted much attention. No commercial FAdV vaccine is licensed in Iran; however, by 2012, FAdV was detected from two broiler breeder flocks (8). Since then, the number of IBH cases has increased. In 2013 and 2014, viruses were isolated from the provinces in the north of Iran, whereas in 2015, FAdV spread to the center of Iran (such as Semnan and Markazi). Vertical transmission of FAdV associated with IBH has been detected (5). Rapid spread of FAdVs in Iran might be related to vertical transmission. Application of strict biosecurity measure in Iranian broiler breeder farms prevent introduction of FAdVs during the rearing period. However, the virus may have infected the flocks in the production phase and resulted in vertical transmission. The high similarity between breeder and broiler FAdVs suggests that the viruses may vertically transfer to the progeny and, consequently, spread laterally among flocks in different parts of Iran.
In this study, phylogenetic analysis of the isolates revealed two FAdV species (D and E) and two serotypes 11 and 8b associated with IBH, in agreement with other reports in Iran (8,15) and other countries. An investigation of the outbreaks of IBH in Australia in 2011 determined IBH caused FAdV-8b or FAdV-11 in a meat breeder and broiler flocks (20). Also, an epidemiologic investigation of outbreaks of FAdV in commercial chickens in Korea indicated that all FAdVs types 8b and 11 had relevance to IBH lesions (1). FAdVs associated with IBH outbreaks in Canada were genetically related to FAdV-2, FAdV-8a, and FAdV-11 (17). In Japan, FAdV-D (11,14) and in Slovenia, FAdV-E serotype 8b (24) was found as the causative agent of IBH. Most of the isolates in Iran belonged to FAdV-D serotype 11, similar to our previous finding in 2012. Sequences of FAdV-D obtained in this study shared 98.4% to 100% nucleotide identity to each other and between 99% and 99.8% identity with the previously published sequences in Iran (8). FAdV-D isolates described in this article have been clustered (II and III). Cluster II had the highest identity to Brazil strain, and Cluster III was similar to strains in Canada, China, India, Korea, and the United States. Sequences of FAdV-E obtained in this study shared 100% nucleotide identity to each other, 97% identity with the previously published sequence in Iran, and 100% nucleotide identity with three strains from Italy, Austria, and Hungary. It is proposed here that the virus might spread from Europe to Iran through imported eggs and chicks. Immunosuppressive agents, such as infectious bursal disease virus (IBDV) and chicken infectious anemia virus (CIAV), are predisposing factors to IBH outbreaks or can exacerbate clinical appearances concurrently with FAdV infections (4,22). Studies in Canada, the United States, and New Zealand indicated that IBH occurred as a primary disease, without association with IBDV or CIAV (3,5,6,12,17). Breeder flocks are vaccinated by chicken infectious anemia and infectious bursal disease virus vaccines in Iran, and as a result, chicks have adequate maternal-derived antibodies to be protected against clinical and subclinical forms of diseases at an early age. On other hand, in a few flocks affected with IBH, CIAV and field IBDV were not detected by PCR or reverse transcriptase–PCR. Based on author’s previously published data (8), IBH most commonly occurs as a primary disease in Iran and needs more attention.

Identification of FAdV serotypes is important in epidemiologic studies of disease outbreaks, the development of preventative measures, and the adoption of vaccination strategies. Nucleotide sequence diversity analysis is a reliable molecular epidemiology method for characterization of FAdV viruses. The current study is the first report on the distribution of FAdVs in Iran besides molecular characterization. It appears that IBH has been recently increasing in meat type chickens in Iran that can result in large economic losses to the poultry industry.

REFERENCES


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