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Phylogenetic analysis of pigeon adenovirus 1 in clinical specimens of domestic pigeons (*Columba livia domestica*) in Iran

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Abstract

An internationally identified syndrome that leads to deaths between domestic and ornamental pigeons, particularly after racing is young pigeon disease syndrome (YPDS). This study was conducted to determine the status of pigeon adenoviral infection and molecularly characterize the pigeon adenovirus in Ahvaz pigeons. Sixty stool samples of healthy pigeons (young pigeons and adult pigeons) and 60 stool samples of diseased pigeons (young and adults) with symptoms of lethargy, weight loss, crop stasis, vomiting and diarrhea were examined. Samples were screened for aviadenoviruses by polymerase chain reaction (PCR) assay and degenerated primers set to target the aviadenovirus polymerase (pol) gene were used which was designed in this study. Screening for pigeon adenovirus 1 (PiAdV-1) was performed using a primer pair that targeted the fiber gene of PiAdV-1. Out of 120 stool samples, six samples (5.00%) were positive for aviadenovirus. The results showed that independent from pigeons' age status, 5.00 and 3.33% of sick and of healthy pigeons were positive for PiAdV-1, respectively. Genomic sequencing revealed that the viruses detected in Ahvaz pigeons belonged to the PiAdV-1 genotype. The results in pigeons revealed a 98.10 - 99.53% nucleotide similarity when compared to other strains of PiAdV-1 (TR/SKPA20, P18-05523-6 and strain IDA4) formerly deposited in GenBank® in Türkiye, Australia and The Netherlands. As far as the authors know, this was the first record of phylogenetic analysis of PiAdV-1 in Iran.

Introduction

Most adenoviruses thought as complicating organisms in diseases are mainly caused by other microorganisms or as components of multifactorial problems. However, some adenoviruses are primary and highly pathogenic such as pigeon adenoviruses.[1] Pigeon (*Columba livia domestica*) adenovirus infection is often found in young pigeons with high losses.[2-5] In Europe, pigeon adenovirus (PiAdV) has previously been described as the leading cause of acute mortality in racing pigeons of all ages.[3,6] Young pigeon disease syndrome (YPDS) causes deaths between domestic and ornamental pigeons, especially after competitions, and is an internationally known health problem. The disease causes several nonspecific symptoms such as diarrhea, vomiting, anorexia and crop stasis, and usually occurs in young pigeons under one year old.[7-10] To date, the cause of YPDS is unclear.

One of the hypotheses regarding the causative agent of this syndrome is the involvement of adenoviruses as a potential cause of YPDS. So far, five genera have been identified in the adenovirus family: *Mastadenovirus, Aviadenovirus, Siadenovirus, Atadenoviru,* and *Ichtadenovirus.* Natural adenoviral infection of pigeons is caused by pigeon adenovirus 1 (PiAdV-1),[2,11] or some serotypes of fowl adenoviruses (FAdV) which all belong to *Aviadenoviruses*.[2,6,12] Two different adenoviral diseases were described in pigeons as type I and type II. Type I adenovirus infection (the classical adenovirus infection) has significant similarities to YPDS because it is found almost exclusively in young pigeons.[3] In type I adenoviral infection, clinical signs are often complicated by *Escherichia coli* infection which causes weight loss, vomiting and watery diarrhea.[1] Type II adenoviral infection affects pigeons of all ages and is determined by large hepatic necrosis and subsequent sudden death,[3] hence, the liver sample is suitable tissue for adenovirus detection in dead pigeons.

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To date, there is little information on the importance, potential role and distribution of PiAdV in pigeons. Considering the importance of adenoviral infections in pigeons and the fact that no previous phylogenetic study has been conducted in Iran, and also considering that many pigeons in the Ahvaz region are referred to the veterinary hospital with the mentioned symptoms, and considering the occurrence of diseases with similar clinical manifestations in pigeons and the importance of differential and rapid diagnosis, the current study was intended to determine the status of pigeon adenoviral infection in Ahvaz and to characterize pigeon adenoviruses phylogenetically in domestic pigeons suspected of adenoviral infection.

Materials and Methods

Sample collection. The study was conducted from March 2019 to March 2020 in Ahvaz, Iran. Here, 120 stool samples were taken from diseased and healthy domestic pigeons. Sterile sheets of paper were placed on the floor of the cages and about 30 min later, using sterile wooden spatulas, fresh stools were gathered from the bed of cages of each bird and then stored in sterile vials separately. They were immediately carried to the laboratory for further processing. Stool samples of 60 diseased pigeons (30 young under one year old and 30 adults over one-year-old) were obtained from pigeons introduced to the department of avian medicine with symptoms of lethargy, crop stasis, weight loss, vomiting and diarrhea. Fecal samples were also taken from 60 clinically healthy pigeons (30 young pigeons under one year old and 30 adult pigeons over one year old) kept in Ahvaz birds pet shops (Table 1). Five diseased pigeons died and since one type of adenoviral infection was determined by large hepatic necrosis and subsequent sudden death, therefore, for detection of pigeon adenovirus in dead pigeons, liver samples were taken from all of them. This study was approved by the Shahid Chamran University of Ahvaz Ethical Commission for Animal Experiments under verification number EE/99.3.02.38352/scu.ac.ir.

DNA extraction from the samples. Using the Genomic DNA Extraction Kit (RahaZist, Ahvaz, Iran) according to the protocol of the manufacturer, DNA was extracted from stools and liver samples. All extraction steps were performed as follow: For stool samples, 30.00 mg of each stool sample was poured into a sterilized microtube. Then, 500 µL of lysis buffer was poured into each microtube containing the samples. For liver samples, 15.00 mg of each liver tissue was dumped into a sterilized microtube. Then 350 µL of lysis buffer containing 1.00% mercaptoethanol was poured into each microtube containing the samples in two steps and the samples were mixed and homogenized. Then the samples were placed in a water bath at 55.00 ˚C for 1 hr. The microtube was removed at 15 min intervals and vortexed for several seconds. Then the microtubes were centrifuged for two min at 14,000 rpm. To a spin column with a collection tube, the supernatant was moved. The spin column was then centrifuged for one min at 7,000 rpm and then for one min at 11,000 rpm. After discarding the liquid inside the collecting tube, 750 µL wash buffer was dumped into the spin column. Then it was centrifuged for two min at 14,000 rpm. After discarding the liquid inside the collecting, the washing step was repeated once more and then the column was centrifuged for three min at 14,000 rpm to dry. The column was intently carried to a new 1.50 mL tube. Afterward, 75.00 µL of 55.00 ˚C preheated elution buffer was dumped into the center of the column and incubated at room temperature for 3 - 4 min. This column was centrifuged for two min at 14,000 rpm. The filtrate was maintained at – 20.00 ˚C and used as the template in polymerase chain reaction (PCR).

Molecular detection of aviadenoviruses, PiAdV-1 and PiAdV-2. For detection of aviadenoviruses, PCR was performed using the primers (the forward primer AvAdV-Pol (F- ATGGGMGCSACSTAYTTYGAYAT) and reverse primer AvAdV-Pol (R- AARTTRTCCCKRAANCCGATVTA) designed in this study to amplify the aviadenovirus polymerase (pol) gene. It created the product with 608 base pairs (Fig. 1A). The PCR was performed using a thermal cycler (Eppendorf, Hamburg, Germany) with the following protocol: Initial denaturation at 95.00 ˚C for 5 min, followed by 40 amplification cycles (95.00 ˚C for 50 sec, 53.80 ˚C for 45 sec, 72.00 ˚C for 40 sec) and a final extension cycle (72.00 ˚C for 5 min). In the final volume of 25.00 µL, the polymerase (pol) gene was amplified using 12.50 µL of 2X master mix (with 1.50 mM $MgCl_2$, 0.20 mM of dATP, dCTP, dGTP and dTTP; Amplicon, Copenhagen, Denmark), 3.00 µL of template DNA, 4.00 µL of primer pair (10.00 µM) and 5.50 µL of PCR grade water. Samples positive for aviadenoviruses were tested for PiAdV-1 and PiAdV-2. The PCRs were performed using a thermal cycler (Eppendorf, Hamburg, Germany). For the detection of PiAdV-1, PCR was carried out using the primers as the forward primer PiAdV-1-F1 (F- ATCAACTACGACAACGAA

Table 1. The number (**%**) of positive fecal samples according to PCR test.

Sample source	Positive aviadenoviruses	Positive PiAdV-1	Positive PiAdV-2
Young diseased pigeons	3/30 (10.00)	2/30 (6.66)	0/30 (0.00)
Old diseased pigeons	1/30 (3.33)	1/30 (3.33)	0/30 (0.00)
Young healthy pigeons	2/30 (6.66)	2/30 (6.66)	0/30 (0.00)
Old healthy pigeons	0/30 (0.00)	0/30 (0.00)	0/30 (0.00)
Total	6/120 (5.00)	5/120 (4.16)	0 (0.00)

GGC) and reverse primer PiAdV-1-F2 (R- CGGTAGAGTTAC GGGGAAATT) designed to amplify the fiber 2 gene described by Raue *et al*.[11] It created the product with 967 base pairs (Fig. 1B). PCR was performed with the following protocol: Initial denaturation at 95.00 ˚C for 5 min, followed by 40 amplification cycles (94.00 ˚C for 1 min, 60.00 ˚C for 1 min, 72.00 ˚C for 90 sec) and a final extension cycle (72.00 ˚C for 10 min). For the detection of PiAdV-2, the forward primer HEX (F- GTAACATGAGCGTGCTGTTTG) and reverse primer HEX (R- CTGAGAAACGAAACCCGAATTG) were designed to amplify the Hexon gene described by Teske *et al*.[13] It created the product with 643 base pairs. The PCR was performed with the following protocol: Initial denaturation at 95.00 ˚C for 6 min, followed by 40 amplification cycles (95.00 ˚C for 45 sec, 47.30 ˚C for 60 sec, 72.00 ˚C for 60 sec) and a final extension cycle (72.00 ˚C for 8 min). A total volume of 25.00 µL of reaction mixture containing 2.00 µL of primer pair (10.00 µM), and 2.00 µL of template DNA, 8.25 µL of PCR grade water, 0.25 $MgCl_2$, and 12.50 µL of 2X master mix were used for detection of PiAdV-1 and PiAdV-2. By electrophoresis of 5.00 µL of the product (which was mixed with 1.00 µL of loading buffer) in 1.50% (w/v) agarose gel with TAE including 100 mM Tris HCl (pH 9.0), 40.00 mM EDTA containing 1.80 µL safe stain (Sinaclon, Tehran, Iran), PCR products were detected and observed by transillumination under ultraviolet (Uvidoc HD6; Uvitec, Cambridge, UK). The size of the amplified products was appraised through comparison with a DNA ladder of 100 bp (Sinaclon).

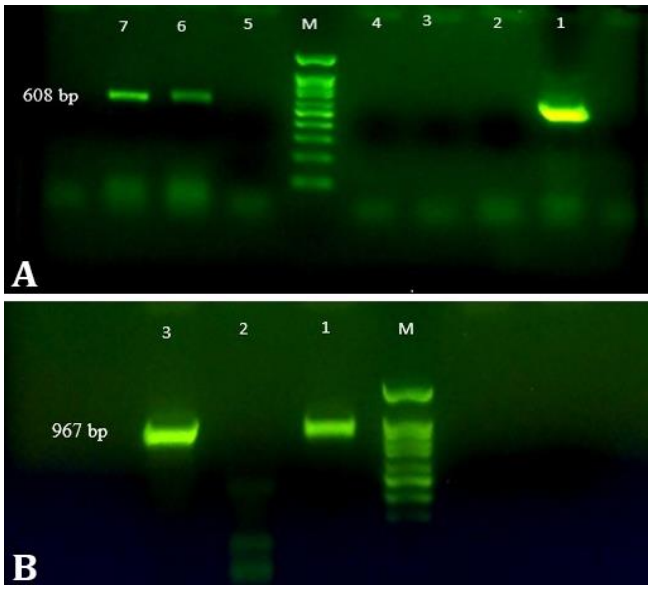

Fig. 1. A) PCR for tracing aviadenoviruses using general primers set to target aviadenoviruses polymerase (pol) gene and examination of products by agarose gel electrophoresis**.** Lane 1: Positive control. Lane 2: Negative control (water). Lanes 3, 4 and 5: Negative samples. Lanes 6 and 7: Positive samples which produced product with a size of 608 bp of aviadenoviruses. Lane M: 100-bp molecular marker (Sinaclon). **B)** PCR for tracing PiAdV-1 using primers set to target the fiber 2 gene and examination of products by agarose gel electrophoresis**.** Lane M: 100-bp molecular marker (Sinaclon). Lanes 1 and 3: Positive samples which produced product with a size of 967 bp of PiAdV-1. Lane 2: Negative control (water).

Sequencing and phylogenetic analysis. To confirm the PCR results, 50.00 µL of PCR product of positive PiAdV-1 samples along with 100 µL of Fiber-F (10.00 pmol) and 100 µL of Fiber-R primers (10.00 pmol) were sent to Gene Fanavaran Co. (Tehran, Iran) for sequencing. Obtained sequence data during this study were deposited to Gen-Bank® under the accession numbers OL442100, OL456215 and OL538251. After converting the sequences to FASTA format, they were aligned and recognized by searching databases using the online system of local alignment tools (BLAST) on National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). ClustalW method by MEGA Software (version 6.0; Biodesign Institute, Tempe, USA) and SnapGene® (version 3.2.1; GSL Biotech, San Diego, USA) were used to compare our sequences with other related sequences in NCBI. With the MEGA Software, by the neighbor-joining algorithm and the Jukes-Cantor distance model, a phylogenetic tree was created according to the nucleotide sequences of the fiber gene. Bootstrap support was evaluated with 1,000 duplicate analyses. The nucleotide similarity of the sequences with the formerly recognized strains is demonstrated in Table 2.

Results

Totally, 120 stool samples were evaluated by molecular assay. In molecular identification of stool samples, out of 120 samples, six samples (5.00% of the total stool samples) were positive in detection of the aviadenovirus genus, of which five samples (4.16% of the total stool samples) were positive for PiAdV-1 genotype (Table 1). Results for the PiAdV-2 genotype were negative. It should be noted that all five liver samples taken from dead pigeons (including three young- and two old- diseased pigeons) were negative for aviadenovirus. In the present study, among birds positive by PCR method, young and old diseased pigeons were presented with signs of lethargy, weight loss, crop stasis, vomiting and diarrhea. Therefore, it seems that gastrointestinal symptoms such as crop stasis, vomiting and diarrhea are common in birds with adenoviral infections. The results showed that regardless of the age of the pigeons, out of 60 stool samples of diseased pigeons, three samples (5.00%) and out of 60 stool samples of healthy pigeons, two samples (3.33%) were positive for PiAdV-1 (Table 1). Regardless of the status of health, out of 60 stool samples of young pigeons, four samples (6.66%) and out of 60 stool samples of old pigeons, one sample (1.66%) were positive for PiAdV-1 (Table 1). Genomic sequencing also revealed that the viruses detected in pigeons belonged to the PiAdV-1 genotype.

Table 2. The nucleotide similarity (%) of the PiAdV-1 sequences in this study with the previously identified strains.

Strain	Host	Country	Accession	Identity with OL442100	Identity with OL456215	Identity with OL538251
Strain IDA4	Pigeon	The Netherlands	FN824512.2	99.30	99.12	99.53
P18-05523-6	Pigeon	Australia	MW286325.1	98.25	98.12	98.48
TR/SKPA20	Pigeon	Türkiye	MN985817.1	98.25	98.10	98.10

Gene sequencing of PiAdV-1 genotype. Phylogenetic analysis showed that three sequences of the present study belonged to the PiAdV-1 genotype. All sequences obtained in this study were deposited to GenBank® under accession numbers of OL442100, OL456215 and OL538251. By the neighbor-joining method and the Jukes-Cantor distance model, phylogenetic trees were built (Fig. 2). Bootstrap support was evaluated with 1,000 duplicate analyses. The results disclosed a 98.10 - 99.53% nucleotide similarity when compared to other strains of PiAdV-1 (strain IDA4, TR/SKPA20 and P18-05523-6) formerly deposited in GenBank® from The Netherlands, Türkiye and Australia (Table 2).[14-16]

Discussion

In this study, performed for the first time in Iran, the results showed that 4.16% of samples were positive for the PiAdV-1 genotype using the PCR method. This indicated that there was virus shedding at the time of sampling. In the present study first, the samples were screened for the aviadenovirus genus. Out of 120 samples, six samples were positive in detection of the aviadenovirus genus, of which five samples were positive for PiAdV-1 genotype, and because the study aimed to examine pigeon adenoviruses, only the samples positive for pigeon adenoviruses were analyzed phylogenetically. Considering that one sample out of the six positive aviadenoviruses samples was negative for PiAdV-1 and PiAdV-2, it might be a novel adenovirus that should be investigated in future studies. Genomic sequencing revealed that the viruses detected in Ahvaz pigeons belonged to the PiAdV-1 genotype. The results disclosed a 98.10 - 99.53% nucleotide similarity when compared to other strains of PiAdV-1 (strain IDA4, TR/SKPA20 and P18-05523-6) formerly deposited in GenBank® from The Netherlands, Türkiye and Australia[14-16] In addition, the sequences of the present study were the most closely related to the strain IDA4 from The Netherlands rather than the strain TR/SKPA20 from Türkiye.[14,15]

So far, there are a few reports of clinical infection of pigeons with pigeon adenoviruses in other parts of the world. Weissenbock and Fuchs examined the liver of 226 pigeons using histopathology to detect herpesvirus and adenovirus.[17] In 4.00% of the samples inclusion bodies were observed of which five pigeons (2.20%) were positive for adenovirus. The reason for the low prevalence of adenovirus infection in the mentioned report seems to be related to the study method. Because the presence of the virus in pigeons is not always associated with pathological lesions, the virus may not be detected by histopathology.

Raue *et al*. performed a comprehensive study on pigeons with or without the prevalence of YPDS.[5] The research comprised checking histories, clinical signs, pathology, microbiological and parasitological analysis. Similar to the present study, PiAdV was not detected in any liver sample. Young racing pigeons infection with fowl adenovirus (FAdV), pigeon circovirus (PiCV) and pigeon herpesvirus (PiHV) are stated repeatedly. The impress of these viruses in the pathogenesis of YPDS is commonly accepted. For the tracing of FAdV, PiCV and PiHV in liver

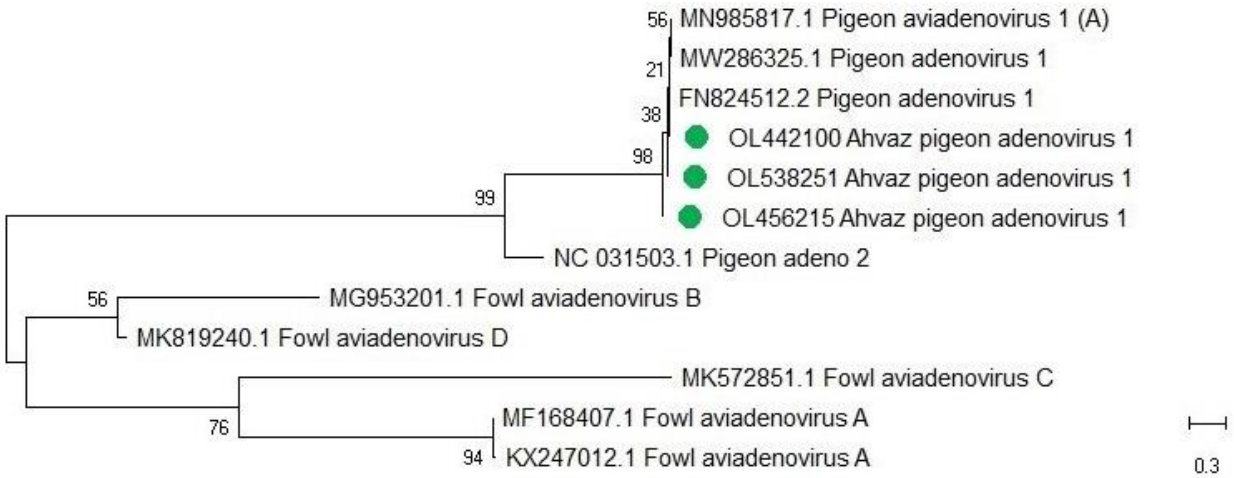

Fig. 2. Comparison of the phylogenetic position of the present PiAdV-1 sequences based on the fiber 2 gene (OL442100, OL456215 and OL538251) with other related strains in the GenBank® database by the neighbor-joining algorithm (MEGA Software).

samples of racing pigeons, Freick *et al*. developed a multiplex PCR.[7] Similar to the present study, aviadenoviruses were not traced in any of the liver samples.[7] According to the results of Vereecken *et al.*, the liver is a more suitable tissue for adenovirus detection and the most important necropsy symptom in dead pigeons caused by adenovirus is related to the liver.[1] According to Freick *et al*.,[7] adenoviruses cause the inclusion bodies in the liver and the amount of viral DNA in liver samples is adequate for detection during the clinical outbreak of infection, therefore, liver samples are particularly useful for the detection of infection.

Catroxo *et al*. surveyed the existence of adenovirus in the stools of free-living pigeons (*Columba livia*).[18] In their study, fresh stool samples of 57 healthy free-living pigeons (50 adults and 7 young birds) were collected in urban areas of Sao Paulo, Brazil and examined by electron microscopy. Among the 57 samples tested, two samples (3.50%) were positive for the presence of adenoviruses. Similar to the results of the current study the prevalence of adenoviral infection was low in the pigeons.

In Poland, Stenzel *et al*.surveyed the epizootic status of some viral infections such as adenoviral infection in different types of domestic pigeons.[9] They studied pigeon adenovirus based on the fiber gene and fowl adenovirus based on the hexon gene. They detected pigeon adenovirus only in 5.00% of young birds with clinical signs of YPDS which was compatible with the results of the current study regarding the low prevalence of adenovirus infection in pigeons. In contrast to the results of the current study, the aviadenoviruses were not detected in any of the studied flocks in the study of Stenzel *et al*.[9] In Germany, Teske *et al*.[13] demonstrated that the PiAdV-2 genotype was broadly spread in racing pigeon lofts (in both healthy and YPDS-affected racing pigeons), while the PiAdV-1 genotype and other aviadenoviruses were not traceable. They reported that independent of the pigeons health situation, about 13.00% of adult and 20.00% of young pigeon flocks harbored the PiAdV-2 genotype. In contrast to the results of Teske *et al*.,[13] in the present study, pigeons were free of the PiAdV-2 genotype. In agreement with the present study, the higher tracing rate in young compared to adult pigeons maybe because of longer and more significant adenovirus shedding in juvenile birds.[19,20] In China, Wan *et al*.[21] reported that a novel adenovirus was spread in pigeons. Respectively, a nucleotide resemblance of 79.00 and 70.90% with PiAd-2 variant A and PiAd-1 was recorded when nucleotide homology analysis of the hexon gene was performed.[21] Also, in the present study, one aviadenovirus was detected which was negative for both PiAdV-1 and PiAdV-2. It might be a novel adenovirus that should be investigated in future studies.

Due to the detection of the virus in young and adult diseased pigeons, the presence of the clinical type I and type II adenoviral disease and also YPDS in the Ahvaz region is possible, however, for definitive confirmation additional studies such as histopathological examinations are required. Through histopathological examinations, it is possible to distinguish between clinical type 1 and type 2 adenoviral diseases. Therefore, in future studies, in addition to virus detection, histopathological studies of the liver should be performed to be able to differentiate the clinical types of the disease.

In the present study, among birds positive by PCR method, young and old diseased pigeons were presented with signs such as lethargy, weight loss, crop stasis, vomiting and diarrhea. Therefore, it seems that gastrointestinal symptoms such as crop stasis, vomiting and diarrhea are common in birds with adenoviral infections. Given the low detection rate of PiAdV-1 in young diseased pigeons, also the detection of the virus in healthy pigeons, there are several possibilities. This situation may indicate that the role of PiAdV-1 as the primary pathogen in pigeons in the occurrence of YPDS is low. Definitive confirmation of this requires additional studies such as histopathological examinations. However, it is likely that PiAdV-1, like other adenoviruses, has the potential to play a role in weakening the immune system.[22] Also, since sampling in pigeons was not performed serially in several different periods, detection of the virus in healthy young pigeons may indicate the existence of a latent period in these birds in which after the incubation period, the virus can also cause clinical signs of adenoviral infections in these seemingly healthy birds. Therefore, in subsequent studies, pigeons should be sampled serially at different time intervals so that if the bird appears to be apparently healthy in a latent state and during the incubation period, subsequent sampling will show the clinical manifestations of the disease that may be considered as a sick bird.

Due to the infection of pigeons in Ahvaz city with the PiAdV-1 genotype, this issue should be considered during clinical examinations of pigeons that have symptoms such as vomiting, acute watery diarrhea, crop stasis, weight loss and sudden death. In particular, due to fewer adenovirus diagnoses and fewer studies in this area, the disease is usually not diagnosed and is mistaken for other diseases, especially Newcastle disease or salmonellosis. According to the present study, since the prevalence of this virus in young birds was higher than in adult birds, young birds were likely more sensitive and excrete the virus for a longer time and this was important in the differential diagnosis of young pigeon diseases in the Ahvaz region.

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Conflict of interest

The authors declare there is no conflict of interest.

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