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Sequencing of pigeon circovirus and the first report of identification of beak and feather disease virus in clinical specimens of domestic pigeons

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Abstract

An internationally recognized syndrome that leads to deaths among domestic and ornamental pigeons, particularly after racing, is young pigeon disease syndrome (YPDS). Pigeon circovirus (PiCV) is regarded as one of the potential factors contributing to the occurrence of YPDS. This survey was conducted to determine the prevalence of PiCV infection and molecularly characterize the PiCV in pigeons suspected of YPDS. Eighty fecal samples were collected from 80 diseased pigeons (exhibiting symptoms such as lethargy, weight loss, crop stasis, vomiting and diarrhea) from 20 lofts in different areas of Ahvaz, Iran. Also, 20 fecal samples were obtained from 20 clinically healthy pigeons. The nested broad spectrum polymerase chain reaction test was done to identify the circovirus, using primers targeting part of the *replication-associated protein* gene with 350 bp, and several positive samples were sequenced. This study showed that PiCV was detected in 86 out of the 100 samples (86.00%). Two types of circoviruses were determined in the samples. One type of the detected circoviruses was PiCV which based on phylogenetic analysis had high genetic similarity with A, B, G and H genotypes of PiCV. The other type of detected circoviruses was closely related to beak and feather disease virus (BFDV) which causes one of the most significant viral diseases in psittacine birds. This is the first report of BFDV identification in pigeons.

Introduction

The *Circoviridae* family contains small unenveloped viruses with icosahedral structures and a circular single-stranded DNA genome, being the smallest known pathogens in animals.[1] Viruses belonging to this family are known to cause deadly diseases in birds and pigs and have been categorized into the *Circovirus* genus.[2] Chicken anemia virus previously classified in the *Gyrovirus* genus in the same family, along with other human single-stranded viruses, such as torque teno virus, has been placed in the new classification in the *Aneloviridae* family.[3] Circoviruses infect a wide range of different birds, containing many wild and domestic birds. This virus isolation in cell culture or embryonated eggs is challenging leading to many unknowns regarding genomics, isolation, pathophysiology, pathogenicity and host range.[4] Columbid circovirus or pigeon circovirus (PiCV), duck circovirus, beak and feather disease virus (BFDV), goose circovirus, canary circovirus, swan circovirus, raven circovirus, starling circovirus, finch circovirus, gull circovirus and pig circovirus types of 1, 2, 3 and 4 are placed in *circovirus* genus of the *Circoviridae* family.[5] Recently, PiCV infections have been recorded worldwide mostly in domestic and wild rock pigeons, *Columba livia*.[1,6] Currently, PiCVs are classified into nine different genetic groups, from A to I.[7] Pigeons of any age are susceptible to this virus; although, it seems that young pigeons are more sensitive to PiCV infection.[8] The genome of PiCV has two main open reading frames (ORFs). The ORF-V1 codes for a replication-associated protein (Rep), and the ORF-C1 codes a capsid protein (Cap).[5,9] The PiCV has been detected in disease outbreaks with great incidence and sometimes also great fatality.[10-13] The PiCV infection was recognized for the first time in 1993 in the United States,[13] and had been considered to be strongly related to young pigeon disease syndrome (YPDS).[6]

The theory was that any pigeon with YPDS symptoms was positive for PiCV, too. Also, quantitative analysis indicated that the viral load of PiCV in specimens taken from sick pigeons with clinical signs was much higher than

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healthy pigeons. These findings were partially confirmed by the quantitative polymerase chain reaction (PCR) test developed by Duchatel *et al.* which showed that the viral load of PiCV was remarkably higher in liver samples obtained from pigeons with YPDS compared to healthy pigeons.[11] The YPDS is a disorder often leading to death in domestic and fancy pigeons, especially after a race or exhibition. Typically, young pigeons under one year old show symptoms of YPDS. The virus in pigeons causes anorexia, weight loss, feather loss, crop stasis, poor race performance, poor growth, dyspnea, severe diarrhea, severe immunosuppression, and secondary infections, ultimately leading to death.[12] The PiCV infection usually causes high morbidity and low mortality; however, the mortality rate can reach up to 100% depending on secondary infections.[10-13] Several data have shown that YPDS is a result of immunosuppression caused by PiCV infection.[14,15] The PiCV infections lead to the destruction of lymphoid tissue in the immune system and are considered as an immunosuppressive factor in pigeons.[14-17] The higher prevalence of co-infections caused by different pathogens in pigeons that tested positive for PiCV infection suggests that this virus can cause immunosuppression.[18,19]

Transportation of large numbers of birds from one geographical area during pigeon races can lead to the spread of the virus among pigeons and be effective in creating recombinant strains, such as the recombinant strain of psittacine circoviruses.[20] The virus transmission occurs horizontally by consumption of contaminated water and feed,[21] as well as inhalation of contaminated materials including feces and feather dust; however, it is also transmitted vertically.[4] Due to the difficulty of observing the principles of biosafety in racing pigeons, this virus occurs worldwide.[22] However, studies on this virus in Iran are limited to a few reports.[23] Beak and feather disease virus causes psittacine beak and feather disease (PBFD). This disease often occurs in young psittacine birds and is characterized by feather loss, dystrophic feathers, and disorders of the beak surface. The disease has been related to immunosuppression and the majority of birds die due to a secondary infection.[24] Acute and chronic infections have been described, with the acute infection occurring in young birds.[25,26]

Considering that many pigeons in Ahvaz, Iran, are referred to the veterinary hospital with symptoms of YPDS and no previous phylogenetic study has been conducted on pigeon circovirus in this region, the occurrence of diseases with similar clinical manifestations in pigeons and the importance of differential and rapid diagnosis, the current study was aimed to determine the status of PiCV infecttion in Ahvaz. Additionally, the purpose of this study was the characterization of PiCVs phylogenetically in domestic pigeons suspected of YPDS, and investigation of the role of pigeon as a possible reservoir for specific circoviruses of other species.

Materials and Methods

Sample collection. This study was conducted from July 2021 to May 2022. In this study, 100 fecal samples were collected from pigeons with YPDS symptoms (diseased pigeons) and apparently healthy pigeons. To collect the samples, sterile sheets of paper were placed on the floor of the cages. After about half an hr, fresh stools were collected from the cages using sterile wooden spatulas. The stools were then stored in separate sterile vials and immediately taken to the laboratory for further processing. To ensure there was no laboratory contamination, all tests were conducted in the Medical Virology Laboratory of Jundishapur University of Ahvaz, Ahvaz, Iran. This laboratory had previously only evaluated human samples and had not tested any animal or bird samples, including avian circoviruses. Therefore, the possibility of laboratory contamination was eliminated. In this study, fecal samples were collected from 20 different lofts in Ahvaz. This included five lofts in the north, five in the south, five in the east and five in the west of Ahvaz. From each loft, five samples were taken. Four of these samples were from under one-year-old diseased pigeons exhibiting YPDS symptoms such as lethargy, vomiting, diarrhea, crop stasis, regurgitation and weight loss. The 5th sample was from an apparently healthy pigeon under one year old. All specimens were kept in sterile vials separately at – 20.00 ˚C until testing. This study was approved by the Shahid Chamran University of Ahvaz Ethical Commission for Animal Experiments with verification number of EE/1400.3.02.9752/scu.ac.ir.

DNA extraction. The DNA extraction from fecal samples was done using the DNP™ kit (SinaClon, Tehran, Iran) according to the manufacturer's protocol. The extracted DNA was placed in 1.50 mL microtubes and kept at – 80.00 ˚C until PCR.

Nested broad spectrum PCR test. A nested broad spectrum PCR using two degenerated primer sets was applied for the detection of PiCV. Primer sets in the 1st PCR were as follows: Sence (F- AGAGGTGGGTCTTCACNHTBAA YAA) and anti-sense (R-AAGGCAGCCACCCRTARAARTCRTC). Primer sets in the 2nd PCR were as follows: Sence (F- AGCA AGGAACCCCTCAYYTBCARGG) and anti-sense (R-ACGATG ACTTCNGTCTTSMARTCACG).[27,28] The primers targeted the *rep* gene with an expected amplicon size of 350 bp. In the final volume of 25.00 µL, the *rep* gene was amplified using 10.00 µL of 2.00X master mix (Amplicon, Odense, Denmark) with 1.50 mM $MgCl_2$, 0.20 mM of dATP, dCTP, dGTP and dTTP, 1.00 µL of template DNA, 0.25 µL of forward primer (10.00 pmol), 0.25 µL of reverse primer (10.00 pmol) and 13.50 µL of distilled water. The PCRs were performed using a thermal cycler (Peqlab, Erlangen, Germany). The optimized cycling protocol for the 1st round of PCR included 5 min of incubation at 95.00 ˚C, followed by 35 cycles each consisting of 94.00 ˚C for 30 sec, 46.00 ˚C

for 1 min and 72.00 ˚C for 1 min, and a final incubation at 72.00 ˚C for 5 min. The cycling protocol for the nested PCR was identical; however, an annealing temperature of 56.00 ˚C was used in this case. By electrophoresis of 6.00 µL of product in 1.60% (w/v) agarose gel with Tris-acetate-EDTA including 100 mM Tris HCl (pH: 9.00) and 40.00 mM EDTA containing 2.00 µL safe stain (Sinaclon), PCR products were detected and observed by Vilber Lourmat Gel Documentation System (Vilber Lourmat, Paris, France). The size of the amplified products was appraised through comparison with a DNA ladder of 100 bp (Sinaclon).

Sequencing and phylogenetic analysis. Nucleotide sequencing of the circovirus amplicons was done by Gene Fanavaran Company (Tehran, Iran). Twenty-four positive products from the 2nd PCR were selected for DNA sequencing and the resulting nucleotide sequences were submitted to the GenBank. After converting the sequences to fast allignment sequence test for application format, they were aligned and recognized by searching databases using the online system of local alignment tools in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). ClustalW method by MEGA Software (version 10.0; Biodesign Institute, Tempe, USA) and SnapGene (version 3.2.1; GSL Biotech LLC, Boston, USA) were used to compare the sequences with other related sequences in NCBI. With MEGA X Software, by the neighbor-joining algorithm and Jukes-Cantor distance model, a phylogenetic tree was created according to the nucleotide sequences of the *rep* gene. Bootstrap support was evaluated through performing 1,000 duplicate analyses.

Results

The results of the present study showed that out of 100 fecal samples, 86 samples (86.00%) tested positive for circovirus and their sequence was confirmed. The PCR was performed with primers targeting the *rep* gene with an expected amplicon size of 350 bp, which confirmed the presence of circovirus in the positive samples (Fig. 1). Out of the 86 positive fecal samples, 70 samples (87.50%) were from diseased pigeons with symptoms such as lethargy, vomiting, diarrhea, crop stasis, regurgitation and weight loss. Ten samples (12.50%) from these diseased pigeons tested negative for circovirus infection. Also, out of the 20 fecal samples taken from apparently healthy and asymptomatic pigeons, 16 samples (80.00%) were positive for circovirus infection; while, four samples (20.00%) tested negative. The rate of positive fecal samples in the four studied areas in Ahvaz was as follows: The west of Ahvaz had 23 positive samples, the south of Ahvaz had 22 positive samples, the north of Ahvaz had 22 positive samples and the east of Ahvaz had 19 positive samples.

Gene sequencing and phylogenetic analysis. Twenty-six positive products of the 2nd PCR were sequenced by Gene Fanavaran Company. All sequences obtained in the present study were placed into GenBank under accession numbers being shown in Table 1. The nucleotide sequences of the viruses detected in this study were compared with each other and their nucleotide similarity was obtained. They were also compared with the sequences belonging to the genotypes previously deposited in the NCBI. Using MEGA X Software, by neighbor-joining method and Jukes-Cantor distance model, phylogenetic trees were built (Figs. 2 and 3). Bootstrap support was evaluated with 1,000 duplicate analyses. The phylogenetic analysis showed that 24 sequenced samples in the present study had high genetic similarity with A, B, G and H genotypes of PiCV. Most of the identified sequences with high similarity were placed next to the sequence from China belonging to the B genotype. Two sequences were placed next to the sequence from Australia belonging to H genotype. Five sequences were placed next to the sequence from Germany belonging to A genotype. One sequence was placed next to the sequence from China belonging to G genotype (Fig. 2). The results indicated that the percentage of similarity between the PiCV sequences in the present study was 93.31 - 99.61%.

Additionally, one sequence was aligned with the sequence from Australia belonging to BFDV and one sequence was aligned with the sequence belonging to BFDV (T strain; Fig. 3). It is important to mention that the two BFDV sequences identified in the present study had 96.39% similarity. One sequence with a 98.59% similarity was placed next to the sequence belonging to T strain. One sequence with 99.12% genetic similarity was placed next to the sequence from Australia.

The results of the present research showed that in the west of Ahvaz, lofts 2 and 5 were simultaneously infected with PiCV and BFDV. However, other lofts in the west of Ahvaz, as well as the lofts in the south, north and east of Ahvaz were only infected with PiCV (Table 2).

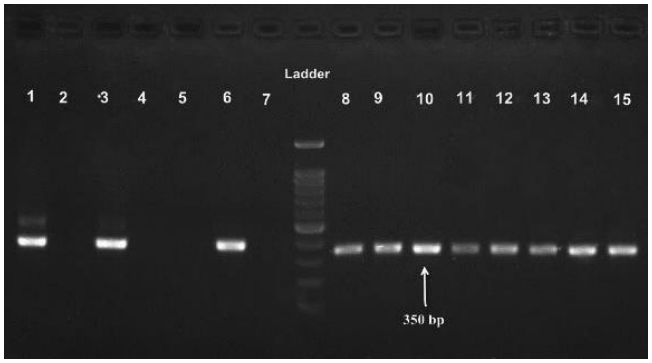

Fig. 1. Polymerase chain reaction for tracing circoviruses using primers set to target *replication-associated protein* gene and examination of products by agarose gel electrophoresis**.** Lane 1: Pigeon circovirus positive control from a previous study; Lane 2: Negative control (water); Lanes 4, 5 and 7: Negative samples; Lanes 3, 6 and 8 - 15: Positive specimens which produced product with a size of 350 bp; Ladder: 100 bp (Sinaclon).

Table 1. GenBank accession numbers for 26 sequences of avian circoviruses in Ahvaz, Iran.

Sequence name	Sequence code	Accession No.	Genotype	Loft No.
PiCV/Iran/Ahvaz-102	102	OM674396	Group A	1
PiCV/Iran/Ahvaz-101	101	OM674395	Group B	1
PiCV/Iran/Ahvaz-99	99	OM674394	Group B	3
PiCV/Iran/Ahvaz-78	78	OM674393	Group B	11
PiCV/Iran/Ahvaz-72	72	OM674392	Group B	12
PiCV/Iran/Ahvaz-11	11	OM674391	Group B	15
PiCV/Iran/Ahvaz-21	21	OM674390	Group A	4
PiCV/Iran/Ahvaz-16	16	OM674389	Group B	6
PiCV/Iran/Ahvaz-49	49	OM674387	Group B	7
PiCV/Iran/Ahvaz-57	57	OM238280	Group H	5
PiCV/Iran/Ahvaz-42R	42R	OM238279	Group G	5
PiCV/Iran/Ahvaz-41R	41R	OM238278	Group B	8
PiCV/Iran/Ahvaz-8	8	OM238277	Group B	17
PiCV/Iran/Ahvaz-3	3	OM238276	Group A	2
PiCV/Iran/Ahvaz-57R	57R	OM125271	Group H	8
PiCV/Iran/Ahvaz-48	48	OM125270	Group B	4
PiCV/Iran/Ahvaz-42	42	OM125269	Group A	5
PiCV/Iran/Ahvaz-20	20	OM125268	Group B	2
PiCV/Iran/Ahvaz-18R	18R	OM125267	Group B	9
PiCV/Iran/Ahvaz-017	017	OM125266	Group B	5
PiCV/Iran/Ahvaz-13	13	OM125265	Group B	10
PiCV/Iran/Ahvaz-23	23	OL840823	Group A	12
PiCV/Iran/Ahvaz-17	17	OL840822	Group B	14
PiCV/Iran/Ahvaz-31	31	OL840821	Group B	20
PBFD/Iran/Ahvaz-pigeon-12	12	OM125264	PBFD	2
PBFD/Iran/Ahvaz-pigeon -14	14	OM674388	PBFD	5

Discussion

In the present study, 26 sequences of avian circoviruses were identified, including 24 sequences of PiCV and two sequences of BFDV. The circoviruses were characterized using partial sequencing of the *rep* gene. There are several diagnostic methods for the circoviruses identification. Serological methods such as hemagglutination test, hemagglutination inhibition test and enzyme-linked immunosorbent assay have traditionally been used for diagnosis. However, these methods have limitations, such as the need for suitable erythrocytes, antigens and antibodies, and their inability to detect interspecies infections. Histological techniques and electron microscopy are also used for diagnosis; but, they are expensive and do not provide information about the genetic variation of the virus.[29] One of the challenges in studying circoviruses is the difficulty in virus isolation, limiting the understanding of its characteristics.[4] Considering these limitations, PCR is a suitable diagnostic method for investigating the prevalence and genetic changes of circoviruses. It is fast, sensitive and affordable, and when combined with virus sequencing, it can reveal the genetic variation of the virus.[30] The present experiment demonstrates that PCR techniques can be used for routine diagnosis and further studies. All circoviruses have two main genes named *rep* and *cap* genes. The *rep* gene is highly conserved and therefore, more suitable as a target gene for diagnostic purposes.[31] Circoviruses have a very wide host range and degenerated primers will be very helpful in the new circoviruses detection.[9,27] Because of the great variability in the circovirus genome sequence, it may not be possible to identify another circovirus from another host species. The nested broad spectrum PCR used in this study succesfully identified BFDV. Therefore, combining this method with PCR sequencing may be useful in circovirus infections identification in animal species where no circovirus species has been defined yet.[27]

In the present study, 86.00% of the pigeons tested positive for circovirus infection. According to Todd *et al.*, circovirus was confirmed by PCR in 84.00% of cases.[15] Raue *et al.* confirmed the presence of circovirus in the liver of pigeons with a rate of 88.00% in Germany using PCR.[6] Zhang *et al.* found PiCV in an average of 75.00% of pigeons in China using a PCR test.[32] Cságola *et al.* identified 57.00% of tested pigeons as a positive for PiCV in Hungary, of which 53.00% were asymptomatic.[22] Examination of diseased pigeons in Slovenia showed that the prevalence of circovirus in pigeons was 74.30%.[33] In a study by Stenzel *et al*. in Poland, more than 70.00% of pigeons tested positive for PiCV by PCR.[19] A study by Mahzounieh *et al.* in Chaharmahal and Bakhtiari province in Iran showed that 24.00% of pigeons were positive for PiCV by PCR.[34] In the aforementioned research, nested PCR was used to identify the PiCV *cap* gene in fecal samples. In the present study, however, nested PCR was used to target and recognize partial sequencing of the *rep* gene. It should be noted that in the current study, the incidence of PiCV

infection in pigeons was higher than that in the study of Mahzounieh *et al*. This difference may be attributed to the fact that Khuzestan is a border province leading to a higher incidence of PiCV infection due to the pigeon trafficking and importation of racing pigeons from other countries. This could be a result of the surveillance lack regarding international trade of pigeons, particularly racing pigeons.

In 2015, serum and tissue samples were collected from six pigeon farms in China. It was determined that 80.70% of sick pigeons and 63.30% of healthy pigeons were positive for PiCV by PCR.[35] The international trade of pigeons, especially racing pigeons, between different countries will facilitate the spread of PiCV. Thus, the possibility of the emergence of new variants increases.[20,36] In the present study, the phylogenetic analysis indicated that 24 samples sequenced had the highest genetic similarity with A, B, G and H genotypes of PiCV. This study also found that PiCV detected in the west, south and north of Ahvaz belonged to different genotypes. Overall, circoviruses were detected at a high rate of 86.00% in this study. The PiCVs detected in some lofts in Ahvaz belonged to different genotypes. This may be caused by the heavy pigeon trafficking and importation of racing pigeons from other countries due to the surveillance lack regarding international trade of pigeons (mainly racing pigeons) in Khuzestan province and Ahvaz.

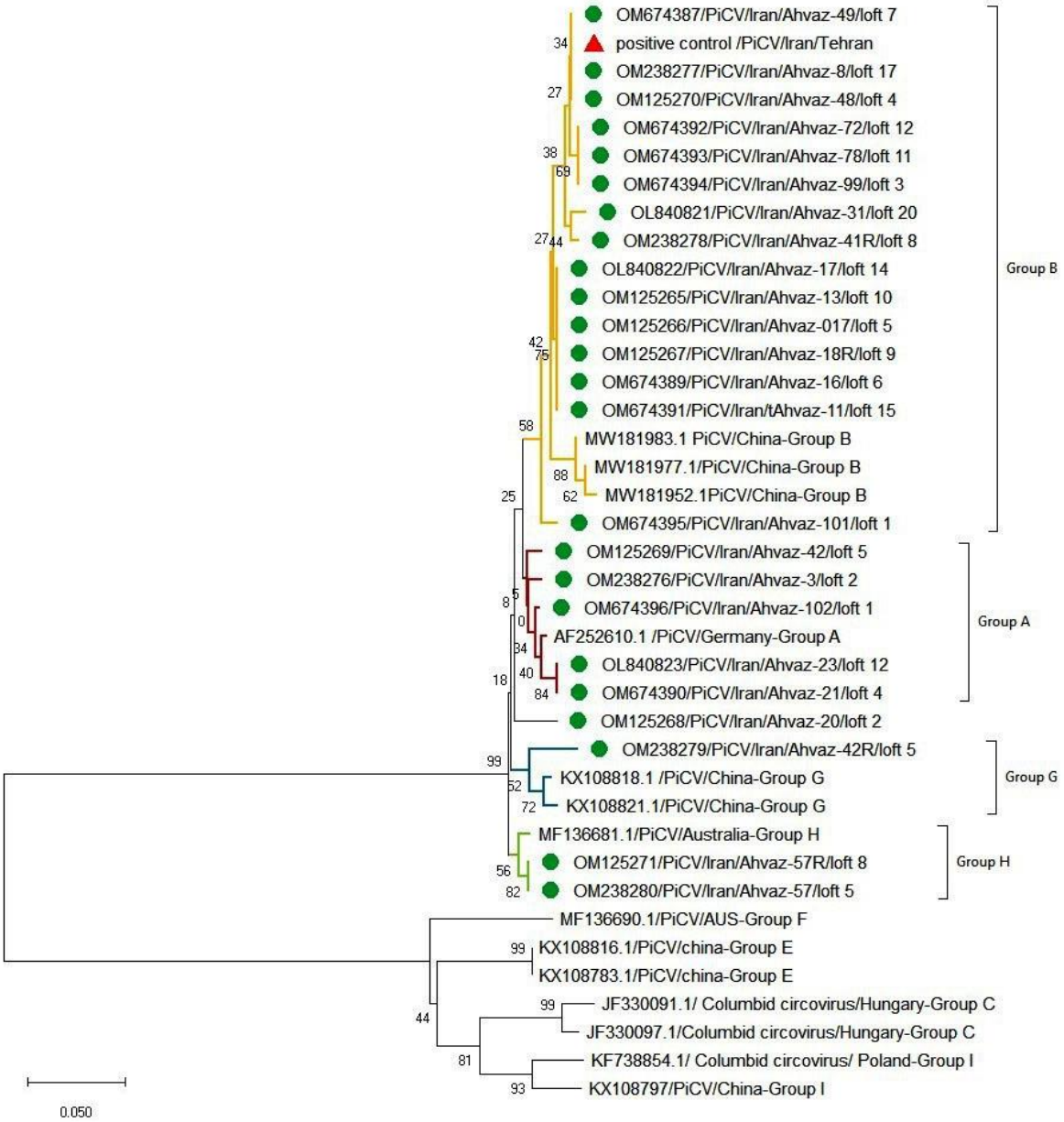

Fig. 2. Comparison of the phylogenetic position of the present pigeon circovirus sequences based on the *replication-associated protein* gene with other related sequences in the GenBank database using the neighbor-joining algorithm (MEGA X Software).

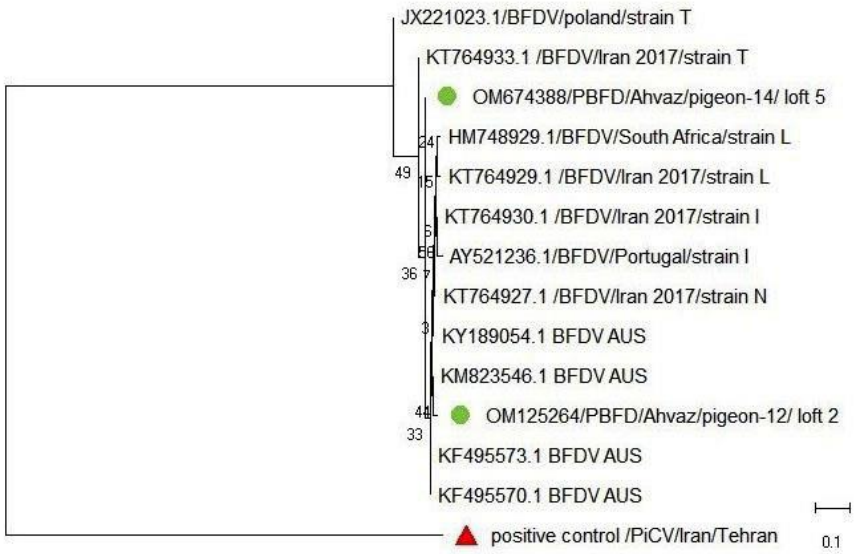

Fig. 3. Comparison of the phylogenetic position of the present beak and feather disease virus sequences based on the *replication-associated protein* gene with other related sequences in the GenBank database using the neighbor-joining algorithm (MEGA X Software).

Table 2. Number of positive birds, investigated areas of Ahvaz and virus genotypes.

Loft Location	Loft No.	Genetic genotypes of the sequenced samples	Positive birds
West	1	A, B	23
	2	A, B, BFDV	
	3	B	
	4	A, B	
	5	A, B, G, H, BFDV	
South	6	B	22
	7	B	
	8	H, B	
	9	B	
	10	B	
North	11	B	22
	12	A, B	
	13	-	
	14	B	
	15	B	
East	16	-	19
	17	B	
	18	-	
	19	-	
	20	B	
Total	20	-	86

In general, competitive events in Ahvaz provide an opportunity for viral recombination, posing a threat to domestic pigeons and allow transmission to wild species. Due to the infection of pigeons in Ahvaz with PiCV, this issue should be considered during clinical examinations of pigeons displaying symptoms such as vomiting, acute watery diarrhea, crop stasis, weight loss, and sudden death. Due to fewer PiCV diagnoses and limited studies in this area, the disease is often misdiagnosed as other diseases, particularly Newcastle disease or salmonellosis.

The PBFD is one of the most important viral diseases in psittacine birds. One significant finding of this study was the detection of BFDV in pigeon samples. The lofts in the west of Ahvaz were infected with this virus. The study detected two sequences of BFDV. One sequence was similar to the sequence from Australia belonging to BFDV and the other sequence was similar to the sequence belonging to BFDV (T strain). It should be noted that only pigeons were kept in the studied lofts and psittacine birds were not present in the lofts. However, the pigeons had a daily flight training program and left the lofts daily; so, the possibility of contamination of pigeons with the droppings of psittacine birds and other wild birds was possible because circoviruses are very resistant to physical and chemical factors[15] and thus, can remain alive for a long time in the environment or even in the digestive system of pigeons. This issue was recently reported in the case of porcine circovirus detected by PCR in goats, as the first inter-species transmission of porcine circovirus,[37] in the case of PiCV detected in other animals, particularly in ticks,[38] and in the case of detection of a novel canary circovirus in a pigeon.[39] There is a discussion about host specificity among circoviruses.[9,29] Therefore, the high similarity between the sequences indicates that these sequences belong to genuine BFDV isolates. Recently a host-switching of BFDV between Psittaciformes and Coraciiformes has been demonstrated.[40] Circoviruses may have a high mutation rate,[27] and the host specificity of circoviruses can be changed by these mutations. Recombinations or viral genome mutations could be another hypothesis for the presence of BFDV in pigeon samples.[6,31,41] Due to the detection of BFDV in pigeons in the Ahvaz region, there is a risk of virus transmission to psittacine birds; so, it is necessary to avoid keeping

psittacine birds alongside pigeons in the Ahvaz region. To the best knowledge of the authors, this was the first report regarding the identification of BFDV in pigeons.

This study showed that circoviruses were detected at a high rate (86.00%) in pigeons. Interestingly, two BFDV sequences were identified in pigeons. The PiCVs detected in most lofts in Ahvaz belonged to different genotypes, which may be caused by the heavy pigeon trafficking and importation of racing pigeons from other countries. This is due to the lack of surveillance regarding the international trade of pigeons, mainly racing pigeons, in Khuzestan province and Ahvaz. According to the results, pigeons can be considered as a possible reservoir or vector for BFDV, causing psittacine beak and feather disease, a fatal disease in parrots.

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Conflict of interest

The authors declare there is no conflict of interest.

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