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Original Article

Molecular Characterization of Psittacine Beak and Feather Disease Virus from Cockatiels (*Nymphicus Hollandicus*) in Iran

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Abstract

Background and Aims: Psittacine Beak and Feather Disease Virus (PBFDV) causes persistent contagious diseases affecting feathers, immune systems, and beaks of companion birds. PBFDV causes progressive beak deformity, plumage loss, and feather dystrophy. We performed the prevalence survey of PBFDV infections in companion birds in the center of Iran.

Materials and Methods: In total, 120 fresh dropping specimens from apparently healthy companion birds were randomly isolated. The dropping specimens were assessed for PBFDV using PCR. Positive samples were sequenced by the Sanger method, and the sequence was approved by alignment and the phylogenetic tree generated by the maximum likelihood technique computationally.

Results: PBFDV was found in 35.8% of specimens. PBFDV was found in cockatiel (*Nymphicus hollandicus*) specimens. Based on phylogenetic analysis, the Iranian isolates had the most similarities to Saudi Arabian isolates.

Conclusion: Such high rates of this pathogen emphasized that healthy *N. hollandicus* in the center of Iran are likely to spread and emergence from PBFDV with subclinical potential. Thus, virological assessments are needed before the export and import of birds.

Keywords: Psittacine Beak and Feather Disease Virus, Phylogenetic analysis, Companion birds, *Nymphicus hollandicus*

Introduction

Psittacine Beak and Feather Disease Virus (PBFDV) causes diseases affecting the feathers and skin of companion birds (1). PBFDV also causes diseases, sudden death, cross-transmission between bird species subclinical course, and imposes severe financial losses on breeders (2, 3).

PBFDV belongs to the *Circoviridae* family and *Circovirus* genus and is categorized as a non-enveloped virus with a single-stranded circular DNA genome in an icosahedral viral capsid. The genome of viruses belonging to the genus Circovirus has two main open reading frames (ORFs) with orientation in the opposite direction (ambisense), one of them encodes the replicase protein (Rep), and another one encodes the immunogenic capsid protein (Cap) (4). The subclinical disease develops in several companion birds, such as parrots and budgerigars. PBFDV was first found in Australia in 1984 in a wild cockatoo. Since then, it was also detected in several species, such as ostriches, canaries, pigeons, geese, ducks, finches, gulls, pheasants, jays, ravens, and starlings (5, 6). There are two genera of *Circovirus* and *Cyclovirus* in the *Circoviridae* family of icosahedrally structured DNA viruses. PBFDV infection is caused by a circo-virus in companion birds and is characterized by the most severe course among young birds (7). It can be transmitted vertically and horizontally and causes high morbidity and low mortality (6). Its mortality rate is associated with the breed, age, and the

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incidence of secondary infections, like chlamydiosis, ventriculitis peritonitis, and mycotic (8, 9).

Table 1. The applied primers in the study, target region, and amplicon lengths

Primer names	Sequence (5′–3′)	Target region	Size (bp)	Reference
PBFDV-F	AACCCTACAGACGGCGAG	*Rep*	717 bp	(13)
PBFDV-R	GTCACAGTCCTCCTTGTACC			

The disease is transmitted by direct contact with the affected birds or by respiratory or gastrointestinal intake of the affected feathers or feces (10).

The typical clinical results are feather loss, abnormal feather growth, immunosuppression, bleeding of feather follicles, and beak anomalies, like enlargement, shiny, or broken. Beak and Feather lesions can be seen together or separately (7, 11). These infections are economically significant because of widespread bird die-offs and risks to breeding potential due to immunosuppression and digestive and vertical tract transmission.

The aim of this study was the molecular detection and phylogenetic analysis of PBFDV in cockatiels in Iran.

Methods

Sampling: We randomly collected 120 fresh dropping swab specimens (*Nymphicus hollandicus*) from apparently healthy companion birds from ten bird sellers in Isfahan Province, Iran. Their age range was 2-12 months, and a swab was obtained from the topmost layer of fresh feces. These new specimens were kept at 4°C until sent to the laboratory. All samples were kept at -20°C in the laboratory until subsequent use.

DNA extraction: Viral DNA was extracted from the dropping samples using a SinaPure DNA Kit (Sinaclon, Iran) based on the manufacturer's instruction. DNA specimens were stored at -20°C until the initiation of molecular testing (12).

PCR: The primers were used for amplifying PBFDV (13) (Table 1). The PCR cycling conditions were as follows: an initial denaturation (96°C for 5 min), followed by 32 cycles of 96°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec. The amplified PCR products were viewed on 2% agarose gel under UV light. When the agarose gel was analyzed, PCR products with a predicted size of 717 bp were detected. No PCR product was obtained in the negative control reactions (Fig. 1).

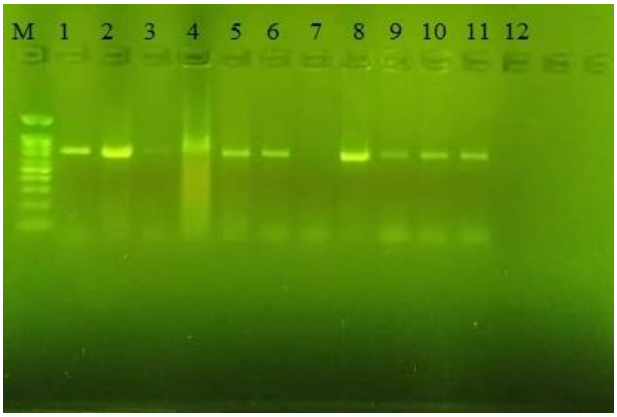


Fig 1. Agarose gel electrophoresis of PCR products. The lanes illustrate molecular weight marker 100 bp (M), positive control (1), positive samples (2, 4, 5, 6, 8, 9, 10, 11) with the 717 bp band, negative samples (3 and 7), and negative control (12)

Sequencing and phylogenetic analysis: Four PCR positive specimens tested in this study were chosen for Sanger sequencing. To purify the PCR products, an AccuPrep® PCR Purification Kit (Bioneer, Korea) was applied. Sequencing was done using the primers (both directions) following PCR (Bioneer, Korea). Through a CLC Sequence Viewer (version 8.0), a phylogenetic tree was constructed using the neighbor-joining technique.

Using sequences from GenBank, the *Rep* gene nucleotide sequences were compared. These sequences were aligned, followed by a comparison with reference strains. Table 2 indicates the strain name, country of origin, accession number, and isolation year of some PBFDV strains. The inclusion criteria for

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sequence analysis represent different positive species.

Table 2. Data for reference PBFDV isolates applied in the current research

Isolate name	Country	Year	Accession number
BVDV-S12	Saudi Arabia	2019	MK803398.1
9IT11	Italy	2014	KF723390.1
BFDV_RS	Republic of Serbia	2014	KJ413143.1
BFDV-J_PL-688_2008	Poland	2008	JX221037.1
PT08	Portugal	2010	EU810207.1
CS15-3981-RCP-WA-2015	Australia	2016	KX449321.1
2NC88B	New Caledonia	2012	JX049216.1
IT24	Italy	2016	JF501527.1

The data relating to reference sequencing obtained from the GenBank database for phylogenetic trees were selected to correspond to the bird's country of origin, the countries that import the birds, and the neighboring countries of Iran. Sequence findings were compared with the reference strains by ClustalW multiple sequence alignments (14) and manually edited using CLC Sequence Viewer 8.0 software. The CLC Sequence Viewer 8.0 program was used to generate a phylogenetic analysis according to the *Rep* gene sequences for PBFDV. The findings were analyzed using bootstrap analysis (1,000 replicates) based on a maximum likelihood method phylogenetic tree (the Kimura 80 method).

Results

PBFDV DNA was detected in 35.8% of samples. The *Rep* gene presentation in the phylogenetic tree showed two groups in Iranian isolates (Fig. 2). According to the tree, the PBFDV positive sequences in the current research, the four of Iran-1, Iran-2, Iran-3, and Iran-4 from *N. hollandicus*, were genetically distinct from the two other PBFDV-positive strains. According to the phylogenetic tree (Fig. 2), Iranian PBFDV isolates fell into two groups:

Group 1: Iran-3 and Iran-4 were similar to isolate with accession number MK803403 from Saudi Arabia.

Group 2: Iran-1 and Iran-2 were in one group with the most similarities.

Such Iranian isolates had more minor similarities to other references strains.

Indeed, the Iranian isolates had the most similarities to Saudi Arabian isolates. Also, Iranian PBFDV isolates showed the minor similarities to European isolates from the Republic of Serbia (accession number: KJ413143), Italy (accession number: JF501527, KF723392), Poland (accession number: JX221006), and Portugal (accession number: EU810207).

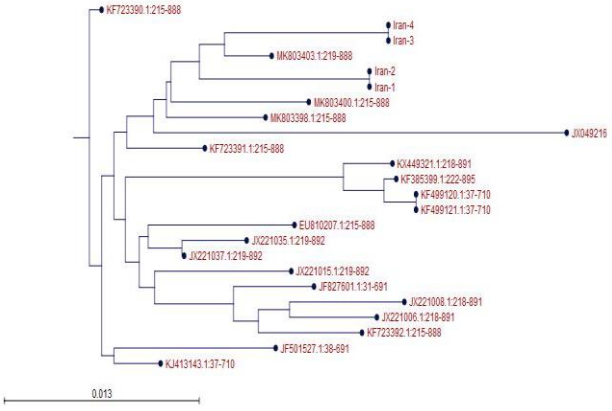


Fig 2. The phylogenetic trees obtained from the nonstructural polyprotein RdRp. Phylogenetic tree indicated a genetic relationship between Iranian PBFDV (Iran-1 to Iran-4) specimens according to nonstructural polyprotein Rep gene based on reference strains.

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In the current research, 43 out of 120 (35.8%) specimens were positive for PBFDV. The species of birds was *N. hollandicus* aged 2 to 12 months. There was no age and positivity correlation in other tested birds.

Discussion

We assessed the occurrence of PBFDV in 120 fresh dropping specimens obtained from companion bird species in the center of Iran. Forty-three PBFD-positive specimens were *N. hollandicus*. More characterization of the isolates of these specimens using PBFDV *Rep* gene-based phylogenetic assessment indicated their close relationship with other positive sequences of different countries, such as Saudi Arabia regarding PBFDV sequences indicating the extensive occurrence of PBFDV infections. PBFDV has been reported in Australia (10), Costa Rica (11), Poland (15), Germany (16), Italy (17), and Taiwan (18), in respectively 31, 19.7, 25.3, 39.2, 8.05, and 41.2% of evaluated specimens. In a study conducted in Turkey, PBFDV (overall detection rate of 19.3% in *Psittacus erithacus* and *Melopsittacus undulatus*) nucleic acids were found in feather specimens isolated from clinically sick birds (12). In the current study, the PBFDV rate was 35.8%, Altan et al. (12) reported PBFDV in feather specimens of *P. erithacus* and *M. undulatus* in western Turkey. The five PBFDV sequences from the previous Turkish study (12) shared two main clusters with South African strains. In eastern Turkey, 113 fresh dropping specimens related to apparently healthy companion birds were randomly isolated and assessed for PBFDV and APV using PCR. PBFDV and APV were found in 48.7% and 23.0% specimens, respectively. The eastern Turkey strains were similar to Iranian isolates (19). Morinha et al. detected PBFDV in 33% of rose-ringed parakeets and 37% of monk parakeets in Southern Spain. The circovirus identified with them is a novel PBFDV genotype, similar to the PBFDV genotypes found in various parrot species kept in captivity in South Africa, China, and Saudi Arabia (20). In the study in China, an epidemic of PBFD struck a farm in Fuzhou, Fujian Province, southeast China, causing the death of 51 parrots. PBFD was diagnosed and using PCR and whole-genome sequencing, the pathogen was recognized. This PBFDV strain resulted in severe disease symptoms and pathological alterations in typical PBFD in parrots, such as feather loss and beak and claw deformities, and severe pathological alterations in different organs. The first documented report of the occurrence of the PBFDV in Iran in seven years old lesser sulphur-crested cockatoos was released by Razmyar et al. (21). In another study in Tehran-Iran, 55 DNA specimens related to nine various species belonging to the order Psittaciformes were tested. They revealed that the Iranian PBFDV were clustered into four genetically certain clades of various genetic subtypes of PBFDV (22). The mutation in circoviruses can occur in their core genomes (23, 24) and tend to be species and region-specific (3). PBFDV were found in feather, blood, visceral organs, and feces using serological and molecular tests (7, 8). Periodic sampling of fecal samples for elucidating the PBFDV shedding resulted in identifying these infections (10). Fecal samples can be easily collected compared to the other samples, particularly in the breeding season, and can be applied for uncovering infections. Therefore, fresh dropping specimens were applicable for investigating both viruses in our research. PBFDV detection according to PCR and serology is expensive when used accurately along with hygiene and quarantine measures. These precautions seem not enough for the removal of the long-term PBFD threat. The infective nature and progressive morbidity of the disease cause the culling of infected birds. However, the culling option is mean-ingless for endangered animals (6, 7).

Adult and fledgling birds are equally at considerable risk because adult birds can carry their infections with no clinical sign (1). There is no effective vaccine against PBFDV infections. A comprehensive health strategy, long quarantine, and screening protocols are suggested in hatcheries to prevent PBFD. Here, we reported the high prevalence of PBFDV in dropping swab specimens of *N. hollandicus*

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birds in the center of Iran. The phylogenetic assessment showed that the PBFDV was placed with the Iranian sequences. The phylogenetic assessment indicated that these diseases are possibly transmitted by imported birds. Thus, early diagnosis, particularly using PCR, is important for protecting healthy birds. In addition, measures, like the elimination of positive birds, routine assessment, and using strict hygiene rules are helpful to control the disease spread with PBFDV agents resulting in economic losses.

Conclusion

Exchange and trade of affected birds and contaminated equipment cause the spread of viruses, which is possibly the cause of similarities between Iranian isolates and Saudi Arabia viruses.

Thus, virological assessments are needed before the export and import of birds. Further studies from both diseased and apparently healthy hosts from different geographical regions further explain the prevalence and risk factors of PBFDV infections in Iran.

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None.

Conflict of interest

The authors declare that they have no competing interests.

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