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Molecular detection and characterization of beak and feather disease virus in psittacine birds in Tehran, Iran

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Summary

Beak and feather disease virus (BFDV), a member of genus circovirus, is a small, non-enveloped, single stranded DNA virus. Although BFDVs are among the most well studied circoviruses, there is little to no information about BFDVs in Iran. The aim of the present study was to detect and identify BFDV molecules from the birds referred to the avian clinic of The Faculty of Veterinary Medicine, Tehran University, Iran. A total of 55 DNA samples were extracted from birds from nine different species of the order psittaciformes. A robust conventional polymerase chain reaction (PCR) was applied to detect the *rep* gene of the virus. Ten out of 55 samples, from four different species, were tested positive for BFDVs in PCR (*Melopsittacus undulates* (4), *Psittacula Krameri* (3), *Psittacus erithacus* (2), *Platycercus eximius* (1)). Molecular identification of the detected BFDVs was performed based on their *rep* gene sequences. The phylogenetic analysis revealed that the Iranian BFDVs from this study were clustered into four genetically distinct clades belonging to different genetic subtypes of BFDVs (L1, N1, T1, and I4). Although the relation between the samples and their related subtypes in the tree are discussed, further studies are needed to elucidate the host specificity and incidence of the BFDVs from different genetic subtypes.

Key words: Beak and feather disease virus, Molecular detection, Psittaciformes

Introduction

Psittacine beak and feather disease (PBFD) is one of the most common viral diseases in parrot species. The causative agent is among the smallest birds' viruses (7-22 nm) and named beak and feather disease virus (BFDV). The virus is non-enveloped and icosahedral, and is composed of an ambisense, circular, non-segmented, single stranded DNA. Circoviral agents convey two major open reading frames (ORFs); ORF V1 is located in a viral strand and encodes the replication-associated proteins (rep), and ORF C1, located in the complementary sense strand, encodes the viral capsid protein (cap). There could be additional small ORFs with unknown functions in some circoviruses (Bassami *et al*., 1998; Maclachlan *et al*., 2011).

Circoviral agents have a great host range. Based on the latest update of the International Committee for the Taxonomy of Viruses (ICTV), the Circoviridae family includes two genera: cyclovirus and circovirus. The genus circovirus contains 22 members, 11 of which are related to bird species; BFDV (Bassami *et al*., 1998), duck circovirus (DuCV) (Hattermann *et al*., 2003), goose circovirus (GoCV) (Soike *et al*., 1999), starling circovirus (StCV) (Johne *et al*., 2006), canary circovirus (CaCV) (Phenix *et al*., 2001), pigeon circovirus (PiCV) (Woods *et al*., 1993), swan circovirus (SwCV) (Halami *et al*., 2008), raven circovirus (RvCV) (Stewart *et al*., 2006), zebra finch circovirus (ZfiCV) (Rinder *et al*., 2015), finch circovirus (FiCV) and gull circovirus (GuCV) (Todd *et al*., 2007). Other circoviruses and circovirus-like viruses also exist that need to be studied to find out whether they should be represented as a separate virus member in ICTV classification or not (Paréand Robert, 2007).

Psittacine beak and feather disease can be presented in three different clinical peracute, acute and chronic forms. Although the disease is characterized by feather and beak lesions, neither clinical findings nor gross lesions are pathognomonic and could vary depending on the host species, age and concurrent secondary infections (Paré and Robert, 2007; Robino *et al*., 2014). Almost all psittaciformes are considered susceptible to this virus. Lymphoid depletion is a common feature of the disease (Todd, 2000). The presence of globular or botryoid, basophilic intracytoplasmic inclusions within different cells in a variety of tissues is considered to be diagnostic, but it is not consistence in all species (Jing *et al*., 2011; Schmidt *et al*., 2015). There has been little success in isolating circoviruses (Mészáros *et al*., 2014), and the diagnosis is based on the demonstration of virus antigens or nucleic acids in clinical specimens or bird tissues. Various molecular techniques are helpful in detecting circoviruses, among which, polymerase chain reaction (PCR)-based ones are the most common and specific methods (Todd *et al*., 2002). They usually target the *rep* gene, as it is more conserved than the *cap* gene for diagnostic purposes (Todd *et al*., 2008; Varsani *et al*., 2011; Julian *et al*., 2013).

The aim of the present study was to detect and

identify BFDV molecules from the birds referred to the avian clinic of The Faculty of Veterinary Medicine, Tehran University, Iran. To the best of our knowledge, this is the first report of BFDVs molecular detection in different species of pcittacine birds in Iran.

Materials and Methods

Sampling

Between October 2014 and April 2015, a total of 55 samples with a variety of clinical manifestations were collected from different species of parrots referred to avian pet clinics in Tehran, Iran Based on the bird's condition and the owners' consent, samples varied from feathers, droppings and blood to internal lymphoid organs (spleen, liver, bursa of fabricius, depending on the case). Samples were immediately frozen at -80°C for further molecular assessment.

The samples belonged to nine different genus and species including *Psittacus erithacus* (19/55), *Psittacus timneh* (2/55), *Psittacula eupatria*s (5/55), *Psittacula krameri* (9/55), *Melopsittacus undulates* (12/55), *Platycercus eximius* (3/55), *Agapornis fischeri* (1/55) and *Ara chloropterus*, *Ara ararauna* (4/55) (detailed information is presented in Table 1).

Extraction

Total DNA was extracted using a High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's directions, with some minor modifications for blood samples. Briefly, an appropriate quantity of each sample (5-10 µL for blood) was transferred into a lysing buffer containing proteinase K. After incubation, the supernatant fluid was used for DNA extraction using the spin column and eluted later.

PCR procedure

To detect BFDV in psittaciformes, a robust conventional PCR protocol (BFDV-PCR) was performed as described by Ypelaar *et al.* (1999). The primers targeted the *rep* gene with an expected size of 717 bps. Reactions were thermocycled as follows: primary incubation at 96°C for 5 min, followed by 32 cycles of 96°C for 30 s (danaturation), 60°C for 30 s (annealing) and 72°C for 90 s (extention). PCR products were then evaluated using electrophoresis in a 2% agarose gel containing RedSafe TM (iNtRON BIOTECHNOLOGY, South Korea). PCR products of the expected length were considered as positive and sequenced for confirmation.

Sequencing and sequence analysis

The DNA sequencing of the target bands was carried out by Bioneer Biotechnology (South Korea). Nucleotide sequences were submitted to GenBank (Table 2). Sequence analysis was performed using a basic local alignment search tool (BLAST), BioEdit (version 7.2.5) and MEGA 6 software (Tamura *et al.*, 2013). A detailed comparative genomic analysis of DNA sequences from this study was carried out using the representative sequences from 27 strains of BFDV based on Varsani *et al*. (2011) and Julian *et al*. (2013). Phylogenetic analysis was carried out using clustal W and the neighbor joining method (Nei and Kumar, 2000) with a bootstrap of 1000 (Tajima and Nei, 1984) using MEGA6 software. GenBank accession numbers of the nucleotide sequences

Table 1: Clinical specimens from different avian species used to detect avian circoviruses using PCR methods

Order	Species	Sample				Number of birds tested (total=55)	Number of positive birds
		Blood (B)	Faeces	Tissue (T)	Feather		
Psittaciformes	*Psittacus erithacus*	15	-	4	-	19	2 (T, B)
	Psittacus timneh	1	-	1	-	2	-
	Psittacula eupatria	2	-	2	1	5	-
	Psittacula krameri	7	-	2	-	9	3 (B=2, T=1)
	Melopsittacus undulates	-	1	11	-	12	4 (T)
	Agapornis fischeri	1	-	-	-	1	-
	Ara chloropterus and *Ara ararauna*	-	4	-	-	4[a]	-
	Platycercus eximius	-	-	3	-	3	1 (T)

[a] Birds were kept together in one cage

Table 2: GenBank accession numbers of circovirus *rep* gene sequences detected in some avian species in Iran

Strain name[*]	Host species	Host name	Accession No.
BFDV-MH-IR-26-Rep-2014	*Melopsittacus undulatus*	Budgerigar	KT764926
BFDV-MH-IR-27-Rep-2014	*Melopsittacus undulatus*	Budgerigar	KT764927
BFDV-MH-IR-28-Rep-2014	*Melopsittacus undulatus*	Budgerigar	KT764928
BFDV-MH-IR-C5-Rep-2014	*Psittacula krameri*	Ring-necked parakeet	KT764929
BFDV-MH-IR-C9-Rep-2014	*Psittacus erithacus*	African gray parrot	KT764930
BFDV-MH-IR-C10-Rep-2014	*Psittacula krameri*	Ring-necked parakeet	KT764931
BFDV-MH-IR-C27-Rep-2014	*Psittacula krameri*	Ring-necked parakeet	KT764932
BFDV-MH-IR-C38-Rep-2014	*Platycercus eximius*	European rosella	KT764933
BFDV-MH-IR-C50-Rep-2014	*Melopsittacus undulatus*	Budgerigar	KT764934
BFDV-MH-IR-C66-Rep-2014	*Psittacus erithacus*	African gray parrot	KT764935

[*] Isolate names were coded according to the scheme: BFDV-MH-IR-'B'-Rep-'C', where BFDV denotes the beak and feather disease virus and 'MH' refers to the author's name (Mohammadreza Haddadmarandi). The two later letters indicate country of origin (Iran), 'B' denotes the sample number, Rep shows the replication part of circoviral genome and the last part shows year of isolation

from this study are presented in Table 2.

Results

PCR detection of BFDV

Ten out of 55 samples from nine different genuses of psittacine species tested positive for BFDV with PCR (18.2%). The positive samples were from various species including, budgerigars (*Melopsittacus undulatus*; 4/12 birds tested, 33.3%), ring-necked parakeets (RNP) (*Psittacula krameri*; 3/9, 33.3%), African grey parrots (AGP) (*Psittacus erithacus*; 2/19, 10.52%) and European rosella (*Platycercus eximius*; 1/3, 33.3%).

BFDV-positive psittacine birds showed different typical features of PBFD. Three out of four budgerigars belonged to breeders with high fledgling and hatchling mortality rates. The other budgerigar had suffered from a concurrent and refractory chronic ulcerative dermatitis (CUD). Two PCR-positive AGPs showed typical feather deformities and alterations related to PBFD with severe leukopenia detected in their CBC test. The European rosella was in a flock with high yearling mortality. Two out of the three RNP positive cases showed remarkable classical signs of the disease including feather disorder and beak necrosis. The remaining RNP positive cases showed no gross feather abnormalities, nevertheless, they were diagnosed with aspergillosis in post mortem investigations.

Phylogenetic analysis of BFDV

A phylogenetic tree was constructed for BFDV strains (Fig. 1). The partial *rep* gene sequences of Iranian viruses clustered into four close major clades belonging to different subtypes of BFDVs.

Nine detected BFDVs were clustered in the same clades with the viruses from similar host species, but the rosella BFDV of the present study (BFDV-IR-MH-C38-Rosella) comprised another clade with an AGP BFDV strain T1, detected in Poland, with 98% identity. All RNP and Budgerigar BFDVs detected in this study were closely related to the L1 and N1 subtypes with 95.2% and 98% identity, respectively. The two AGP strains in the present study clustered in one clade with strain I4 from Portugal (Varsani *et al*., 2011; Julian *et al*., 2013).

Discussion

In the present study, 10 strains of avian circoviruses were detected in five different avian species using a previously established conventional BFDV-PCR (Ypelaar *et al*., 1999). The detected circoviruses were characterized using partial sequencing of the *rep* gene.

Several diagnostic methods have been developed to detect circoviral agents. Serological tests like heamagglutination (HA), heamagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) have been shown to have limitations such as finding the suitable erythrocyte, antigen or antibody, and were therefore, not reliable for cross species infection diagnosis (Johne *et al*., 2004; Stewart *et al*., 2006; Shearer *et al*., 2009). Histology and electron microscopy (EM) have been applied to detect circoviruses, but require special equipment and expertise (Rampin *et al*., 2006). The laboratory isolation of avian circoviruses is also difficult if not impossible and might cause some restrictions using other diagnostic techniques (Mészáros *et al*., 2014). On the other hand, molecular methods have less limitations, are fast and sensitive, and have shown good results. Therefore, they are more practical for showing incidences and genetic diversity and are the most promising techniques of diagnosing BFDV infections (Khalesi *et al*., 2005). The present experiment

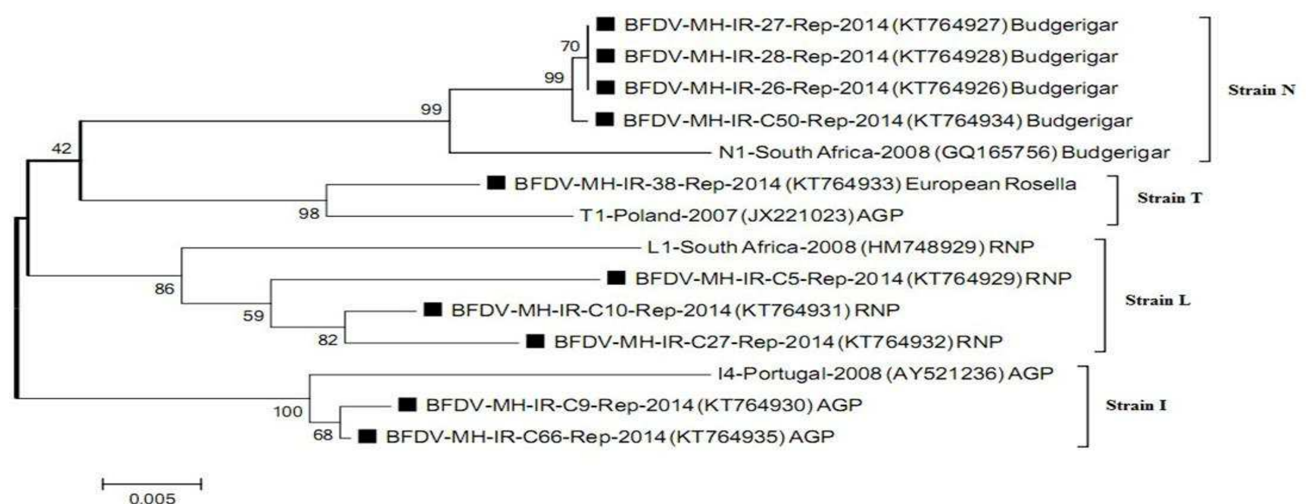

Fig. 1: Neighbour-joining tree of *rep* gene partial sequences of different BFDV strains. The Iranian BFDVs (IR) are marked with a black square and named according to the scheme: 'BFDV'-MH-IR-'B'-Rep-'C', where 'BFDV' denotes the species of the circovirus, 'MH' refers to the name of the author (Mohammadreza Haddadmarandi), the two later letters indicate the country of origin (Iran), the 'B' denotes the sample number, the Rep shows the replication part of circoviral genome and the last part shows the year of isolation, (GenBank accession number) and host species. The other isolates are represented by the name of the strain-subtype, country, and year of isolation (GenBank accession number) of the host species. To avoid complexity, the figure only presents the nearest sequences to ours among 27 strains of BFDV based on Varsani *et al*. (2011) and Julian *et al*. (2013)

shows that the applied PCR techniques can be used for routine diagnosis and further studies.

All circoviruses have two major reputed genes named *rep* and *cap* genes. The *rep* gene has been found to be highly conserved and thereupon more suitable for being as a target gene for diagnostic purposes (Rahaus and Wolff, 2003; Ritchie *et al*., 2003; Bert *et al*., 2005; Hughes and Piontkivska, 2008).

Psittacine beak and feather disease is the most studied circovirus infection in birds and the causative agent, BFDV, seemed to be the most important viral disease in psittacines. In the present survey, 18.2% of the psittacine birds were tested positive for circoviral infections. The single positive budgerigar with the CUD sign supports the hypothesis regarding the role of the virus in such circumstances (Schmidt and Lightfood, 2006).

Different studies showed the variable prevalence of BFDV infections around the world ranging from 2.79% in New Zealand to 45% in the United Arab Emirates, that is, 2.79% in New Zealand (Ha *et al*., 2007); 3.5-4% in the USA (de Kloet and de Kloet, 2004); 8% in Italy (Bert *et al*., 2005); 20.57% in Poland (Julian *et al*., 2013); 23% in Australia (Khalesi *et al*., 2005); 40.4% in Germany (Rahaus and Wolff, 2003); 41.2% in Taiwan (Hsu *et al*., 2006) and 45.13% in the UAE (Hakimuddin *et al*., 2015).

International parrot trades, whether legal or illegal, were a matter of concern in these studies. Trading and trafficking could potentially facilitate the spread of BFDV and the development of new variants of the virus (Varsani *et al*., 2011; Julian *et al*., 2013) and ultimately endanger wild parrot populations throughout the world (Sarker *et al*., 2013).

All budgerigar circoviruses in this study were grouped together in one clade with the N1strain detected in South Africa. Strain "N" has been detected in budgerigars of South Africa and Japan and seemed to be highly species-specific as it has been exclusively detected in budgerigars (Varsani *et al*., 2011).

The ring-necked parakeet (*Psittacula krameri*) was the only endemic parrot from Iran in the present study with a BFDV-positive result (Mansoori, 2013). These detected circoviruses were placed in a single cluster with another RNP circovirus, strain L1 from South Africa. Hence, this strain appeared to show host specificity but as shown in this study, it is no longer limited to a specific region (Varsani *et al*., 2011).

Two strains from AGPs in this study were closely related to strain I4 related to an AGP from Portugal. The strain "I" generally has a broad host range (*P. erithacus* and *Poicephalus* spp.) and has been detected in various geographical regions (Varsani *et al*., 2011).

The only positive circovirus from rosella was very close to strain T1 from Poland. The "T" strain has shown a broad host range in Poland including AGPs, budgerigars and the orange-winged Amazon (Julian *et al*., 2013).

Host specificity of different BFDVs is still debatable. The idea of a strict co-evolution of circoviruses with their hosts has been brought up by Johne *et al*. (2006) and Halami *et al*. (2008). Some studies have revealed a tendency of BFDVs to be species-specific and regionally exclusive (Bassami *et al*., 2001; Ritchie *et al*., 2003; Raue *et al*., 2004). In contrast, some strains were detected in different host species (de Kloet and de Kloet, 2004; Varsani *et al*., 2011; Julian *et al*., 2013). It seems that the constant movement of birds across geographical borders either through trade or natural migration may have played a significant role in the distribution of circoviruses. In addition, mutation and recombination within strains could have caused further complications in declaring BFDVs host adaptations (Heath *et al*., 2004; Varsani *et al*., 2011). Therefore, further investigation is needed in different geographical regions to elucidate the host or geographical adaptation of BFDVs.

Although the aim of this study was not to investigate the prevalence of BFDVs, the relatively high detection rate of the agent highlights the need for further studies with larger sample sizes from both diseased and apparently healthy hosts from different geographical regions to further explain the prevalence and risk factors of BFDV infections in Iran.

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