FREQUENCY OF BEAK AND FEATHER DISEASE VIRUS IN CAPTIVE HEALTHY GREY PARROTS (*PSITTACUS ERITHACUS*) IN PORTUGAL

Gonçalo Portela[1,2,3,4,5]Mário Nóbrega[2,6], Tiago Santos[2], Ana Terraso[7], João Ribeiro Lima[1], Cátia Marques[1,8,9], Margarida Alves[1,5,10]

[1]Faculty of Veterinary Medicine, Lusófona University (FMV-ULusófona), Campo Grande 376, 1749-024 Lisbon, Portugal; [2]ExoticVets, 2670-345 Loures, Portugal; [3]Department of Veterinary Sciences, School of Agrarian and Veterinary Sciences (ECAV), University of Trás-Os-Montes and Alto Douro (UTAD), Quinta de Prados, 5001-801 Vila Real, Portugal; [4]Veterinary and Animal Research Center (CECAV), AL4Animals, University of Trás-Os-Montes and Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal; [5]Animal and Veterinary Research Center (CECAV), Faculty of veterinary Medicine, Lusófona University-Lisbon University Centre, Campo Grande 376, 1749-024 Lisboa, Portugal; [6]Egas Moniz School of Health & Science, 2829-511 Almada, Portugal; [7]Inove Gene, Praceta Horta do Bispo 8B, 7005-259 Évora, Portugal; [8]CIISA - Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Ajuda, 1300-477, Lisbon, Portugal; [9]AL4AnimalS - Associate Laboratory for Animal and Veterinary Sciences; [10]CBIOS - Research Center for Biosciences & Health Technologies, Lusófona University, Campo Grande 376, 1749-024 Lisbon, Portugal.

[*]Corresponding author: goncalo.oliveira.portela@gmail.com

Abstract: *Beak and feather disease virus (BFDV) is responsible for the psittacine beak and feather disease, which affects Psittaciformes, including the Grey Parrot (Psittacus erithacus). Captive birds can act as reservoirs of BFDV, which may have important implications for wildlife endangered species and vice versa. Updated knowledge about the local BFDV frequency and breeders' attitudes towards this disease is paramount to create strategies to reduce BFDV dissemination. The main aim of this study was to estimate the frequency of BFDV in asymptomatic captive P. erithacus kept in Portugal. Blood samples were collected from 100 asymptomatic P. erithacus kept in ten private bird collections from mainland Portugal. After DNA extraction, the presence of BFDV DNA was assessed by nested PCR. Information regarding the bird's living conditions was also collected. BFDV DNA was detected in 8 samples, accounting for a frequency of 8%. Notably, 60% (6/10) of the private bird collections had at least one BFDV positive bird. This study brings new and updated information about the captive fauna of P. erithacus in Portugal. The detection of 8% asymptomatic positive birds highlights the importance of captive birds as reservoirs of this virus. Furthermore, the high number of BFDV affected collections is also a concern regarding BFDV dissemination.*

Keywords*: Beak and Feather Disease Virus (BFDV), Psittacus erithacus, Psittacine Beak and Feather disease.*

1. INTRODUCTION

The beak and feather disease virus (BFDV), belonging to the family Circoviridae, is a virus that affects bird species of several genera (Raidal and Peters 2018; Harkins et al. 2014; Peters et al. 2014). BFDV is the causative agent of psittacine beak and feather disease (PBFD) and it was definitively identified in the late 1980s (Fogell et al. 2016; Bert et al. 2005). This virus is endemic in Australia, and it is currently one of the most clinically relevant diseases of viral origin, especially in Psittaciformes, representing a major threat to biodiversity, being classified as a "key threatening process to biodiversity" (Kessler et al. 2020; Martens et al. 2020a).

This non-enveloped virus, with a diameter of 7 to 22 nm, has a spherical or icosahedral capsid and a genome with a circular deoxyribonucleic acid (DNA) chain with 1993 to 1996 nucleotides and two main opening reading frames (ORF). ORF C1, located in the complementary sense chain, encodes a capsid protein. ORF V1, located in the parallel sense chain, encodes another protein associated with viral replication (Ma et al. 2019; Haddadmarandi et al. 2018; Raidal and Peters 2018; Regnard et al. 2017; Das et al. 2014; Peters et al. 2014; Sarker et al. 2014; Phalen 2006).

BFDV transmission has been shown to occur horizontally and vertically, making it difficult to eradicate the virus from collections of infected birds under human care (Harkins et al. 2014). The horizontal route is considered the most common route of BFDV dissemination, which occurs through inhalation or ingestion of shed viral particles from faeces, feather dust, or crop secretions (Desingu and Nagarajan 2022; Phalen 2006; Bert et al. 2005; Gerlach 1994). It can occur through direct transmission (bird to bird) or indirect transmission (bird to environment to bird) (Haddadmarandi et al. 2018). As for vertical transmission, it has been demonstrated by Rahaus and colleagues (2008), that described a 20% infection rate in embryonated eggs from two Psittaciformes species. Nevertheless, horizontal transmission through environmental contamination also seems to be an important route of infection in young birds (Bert et al. 2005).

After infection, the incubation period of the virus is at least 21 days but may vary depending on the influence of certain factors such as the viral load, the route of infection, the age of the bird, the stage of growth or moulting and the immune status of the infected animal (Desingu and Nagarajan 2022; Khalesi 2007). For young birds, the incubation period is 15 to 30 days, unlike adult birds which may experience an incubation period from months to years

(Phalen 2006). The disease can have three clinical presentations or phenotypes, classified as hyperacute, acute, and chronic (Haddadmarandi et al. 2018; Regnard et al. 2017; Greenacre 2005; Gerlach 1994). However, some authors also consider subclinical as an additional presentation (Philadelpho et al. 2022; Ahaduzzaman et al. 2022; Haddadmarandi et al. 2018; Araújo 2011; Gerlach 1994).

The immunosuppression characteristic of this disease occurs due to virus invasion of lymphoid tissues and consequent necrosis of lymphoid organs (Araújo 2011; Phalen 2006). The necrosis of the thymus, bursa cloacalis, and circulating leukocytes will then lead to an immunosuppressive condition of varying degrees, creating a gateway for opportunistic pathogens, facilitating the emergence of secondary infections, which can then lead to sepsis, often resulting in the death of the birds (Raidal and Peters 2018; MacLachlan and Dubovi 2017; Pendl and Tizard 2016; Allgayer and Pereira, 2014; Harkins et al. 2014; Phalen 2006; Pyne et al. 2005). Generally, the younger the bird, the more severe the immunosuppression. This severity is associated with the fact that birds develop their antibodies in the bursa cloacalis in the first 3 to 4 weeks of life (Desingu and Nagarajan 2022; Pyne et al. 2005). It can also be related to the route of transmission and concomitant infections (Haddadmarandi et al. 2018; Raidal and Peters 2018; Allgayer and Pereira 2014). Particularly in Grey Parrots (*Psittacus erithacus*), the action of the virus on the bone marrow can lead to the onset of severe anaemia, leukopenia, and marrow atrophy and its subsequent replacement with adipose tissue (Pendl and Tizard 2016).

Although PBFD is characterised by beak and feather lesions, clinical signs may vary according to its severity and disease presentation (Haddadmarandi et al. 2018; Allgayer and Pereira 2014; Harkins et al. 2014). The hyperacute presentation usually occurs in neonatal birds and includes clinical signs compatible with septicaemia associated with pneumonia, enteritis, rapid weight loss, and death (Allgayer and Pereira 2014; Greenacre 2005). The acute presentation is characterised by abnormalities in feather development, depression, necrosis and fracture of feathers, haemorrhages, premature moult of affected feathers, stasis of the crop, and diarrhoea (Allgayer and Pereira 2014; Gerlach 1994). Clinical signs associated with the chronic presentation are delayed moulting, feather fractures, deformations such as short, curled, deformed feathers with constriction rings or appearance of stress lines; haemorrhages; hyperkeratosis and feather retention (Fogell et al. 2016;

Phalen 2006; Pyne et al. 2005). Beak lesions occur due to the presence of the BFDV in the germinal epithelium and may lead to hyperkeratosis and overgrowth that may result in the appearance of rhinotheca fissures (Portas et al. 2017; Phalen 2006).

Typical clinical signs can support the hypothesis of BFDV infection; however, clinical signs alone are insufficient to establish a definitive diagnosis (Phalen 2006). The virus can be detected in the blood, even if the animal shows no clinical symptoms (Phalen 2006). Polymerase Chain Reaction (PCR) is the most sensitive technique to detect low viral loads (Ahaduzzaman et al. 2022; Pyne et al. 2005). Since the BFDV genome sequence varies up to 16%, primers must be designed in a conserved region (Phalen 2006). Sequence variations occur mostly in ORF C1, as the capsid protein shows a nucleotide sequence conservation of 76.5% to 83.3%, compared to the replication protein encoded by ORF V1 that achieves amino acid sequence conservation values between 86.9% and 98.3%. For this reason, ORF V1 is chosen for primer design and PCR diagnosis (Raue et al. 2004).

BFDV genome recombination is common and evolutionary (Haddadmarandi et al. 2018; Raidal and Peters 2018; Fogell et al. 2016; Jackson et al. 2014; Massaro et al. 2012). The recombination of different genomes can result in an accentuation of BFDV virulence, which can lead to an increased mortality of birds infected by the new strains (Jackson et al. 2014). This genetic variation may be related to the host species' geographical distribution or the breeding location under human care (Haddadmarandi et al. 2018; Fogell et al. 2016; Massaro et al. 2012). Breeding centers of several bird species present themselves as a risk for the occurrence of these recombinations, as they promote close contact between different bird species and, therefore, different BFDV strains (Jackson et al. 2014).

Presently, due to the high spread rate of the virus, this disease is described in more than 70 species of Psittacidae, either under human care or in wild populations (Fogell et al. 2016). Among these species, 50 are classified by the International Union for Conservation of Nature (IUCN) as "Least Concern", 7 as "Near Threatened" and about 20 are categorised as "Threatened", including *P. erithacus* (Fogell et al. 2016). This spread is primarily caused by the international bird trade (Jackson et al. 2014), but some authors hypothesise the possible action of climate change to extend BFDV distribution (Ortiz-Catedral et al. 2022). Notably, a previous study about the global movement of BFDV points to Portugal as a key location for the

dissemination of the virus in the past (Harkins et al. 2014). Nevertheless, updated data about BFDV in Portugal is lacking.

Asymptomatic carriers of the virus represent a great risk since they do not show early signs of infection, facilitating the spread of BFDV (Phalen 2006). The situation of endangered bird species is particularly delicate, as the virus represents a threat to their survival (Ahaduzzaman et al. 2022; Ortiz-Catedral et al. 2022; Das et al. 2020; Raidal and Peters 2018). Studies have also shown that birds that do not belong to the Psittacidae family can be infected by BFDV (Amery-Gale et al. 2017).

This study aimed to evaluate the presence of asymptomatic BFDV infection in captive healthy *P. erithacus* in mainland Portugal and to characterise the birds' environmental and management conditions.

2. MATERIALS AND METHODS

2.1 Sample collection

Blood samples were collected in early 2022 from healthy captive adult *P. erithacus* belonging to 10 breeders in mainland Portugal (Fig. 1). From each breeder, ten birds were enrolled in the study, accounting for a total of 100 studied birds. Only breeders with bird collections that included at least 10 adult birds of the species *P. erithacus* were sampled. Birds of both sexes (*n*= 50 males; *n*= 50 females) were included and no restrictions on the size (total number of birds) or composition (number or type of bird species) of each collection were considered. Breeders with bird collections with known BFVD infected birds or birds with clinical signs suspected of BFVD were excluded from the study.

Blood samples were collected from the birds' right jugular vein to ethylenediaminetetraacetic acid (EDTA) tubes and stored at -80°C prior to DNA extraction.

The present study was approved by the Ethics Committee and Animal Welfare (CEBEA) of the Faculty of Veterinary Medicine of Lusófona University. At the time of sample collection, an informed signed consent was obtained from each breeder. Furthermore, an epidemiological questionnaire was carried out at each breeder concerning the health status of the included birds, previous BFDV testing, and their housing and handling conditions.

Fig. 1 Geographic location of the breeders enrolled in this study. Light red indicates breeders with one BFDV-

positive *P. erithacus* in their bird collection. Dark red indicates breeders with two BFDV-positive *P. erithacus* in their bird collection. Grey indicates breeders without BFDV infected *P. erithacus.*

2.2 DNA extraction and quality control

DNA was extracted from whole blood samples using the commercial Invisorb® Spin Universal Kit (Invitek Molecular, Berlin, Germany) according to the manufacturer's instructions.

To gauge and ensure the quality and integrity of the extracted DNA, specific primers (12S rDNA L and 12S rDNA H; Table 1), amplifying a 436 bp fragment of the *P. erithacus* 12S rDNA housekeeping gene, were used as previously described (Bert et al. 2005). Briefly, amplification was performed by conventional PCR in a total volume of 25 μL, in individual PCR tubes, composed of 1× MyTaq Reaction Buffer (Bioline®, London, UK), 7 pmol of 12S rDNA L primer, 7 pmol of 12S rDNA H primer, 1 U of MyTaq DNA polymerase (Bioline®, London, UK), between 5 μL to 10 μL of total DNA and sterile deionized water to make up the final volume. Non-template (using sterile deionized water instead of DNA sample) and positive (with *P. erithacus* genomic DNA) controls were included to validate the results of each reaction. The PCR amplification was performed in a BIO-RAD T100™ Thermal Cycler (Hercules, CA, USA), according to the following conditions: 1 denaturation cycle at 94 ºC for 4 min., followed by 35 cycles at 94 ºC for 30 secs., 55 ºC for 30 secs. and 72 ºC for 45 secs., and a final extension cycle of 72 ºC for 7 min. PCR products were visualised on a 2% (w/v) agarose gel electrophoresis.

2.3 BFDV detection

BFDV testing was performed only on samples where the housekeeping gene detection was possible to avoid false negative results due to poor quality DNA or the presence of PCR inhibitors.

To ensure high specificity and sensitivity, a nested PCR was performed using previously published primers (Tomasek et al. 2008). The first round of the nested PCR was performed using primers PBFD 2 and PBFD 4 which amplified a 718 bp fragment of ORF V1 between nucleotide positions number 182 and 899 of the BFDV genome (Table 1) (Tomasek et al. 2008). For this PCR, a reaction mixture was prepared with a total volume of 25 μL, in individual PCR tubes, composed of 12.5 μL of NZYTaq II 2x Green Master Mix (NZYTech®, Lisbon, Portugal), 7 pmol of each primer, 2 μL of DNA and sterile deionized water to make up the final volume. Non-template and positive controls (with the addition of DNA from a known positive sample) were included to validate the results. The PCR amplification was

performed in a BIO-RAD T100™ Thermal Cycler (Hercules, CA, USA), according to the following conditions: 1 denaturation cycle at 94 ºC for 4 min., followed by 35 cycles at 94 ºC for 30 secs., 58 ºC for 30 secs. and 72 ºC for 45 secs., and a final extension cycle of 72 ºC for 7 min.

For the second round of nested PCR, primers PBFD 251 and primer PBFD 609 were used, amplifying a fragment of 359 bp, between position 251 and 609 of the BFDV genome (Table 1) (Tomasek et al. 2008). A reaction mixture with a total volume of 25 µL was prepared, in individual PCR tubes, consisting of 12.5 µL of NZYTaq II 2x Green Master Mix (NZYTech®, Lisbon, Portugal), 7 pmol of each primer, 2 µL of product from the first round of nested PCR and deionized water until the final volume was made up. The amplification of the second round of nested PCR was carried out in the same thermal cycler according to the following conditions: 1 denaturation cycle at 94 ºC for 4 min., followed by 32 cycles at 94 ºC for 30 secs., 67 ºC for 20 secs. and 72 ºC for 30 secs., and a final extension cycle of 72 ºC for 7 min. PCR products were visualised on a 2% (w/v) agarose gel electrophoresis.

To avoid contamination of the samples with exogenous DNA, all good practices, and standard procedures for molecular biology benchwork were adopted.

2.4 Statistical analysis

Fisher's exact test was used for comparisons between groups with an α value of 0.05.

3. RESULTS

3.1 Studied Population

A total of 100 adult *P. erithacus* were studied, belonging to 10 private breeders. All the bird collections included several avian species besides *P. erithacus*. Regarding the environmental conditions and management of these bird collections, it was not possible to determine the number of existing birds or the different species present in each one. This information was difficult to access due to the frequent entry and exit of birds from each collection. Available information about the housing conditions of the studied birds was registered (Table 2).

Most breeders kept the birds under the same conditions (60%, 6/10), namely: without contact with soil and faeces, without direct contact with other avian species, housed in couples and in cages located in the exterior (Table 2).

Overall, 80% (8/10) of the breeders kept the birds with no direct contact with soil and faeces. As for the type of

environment, only two breeders (breeder-4 and breeder-5) kept birds indoors without ventilation, while the remaining 80% (8/10) kept birds in outdoor enclosures. Although all breeders owned other species of birds, only one (breeder-9) allowed the studied *P. erithacus* animals to have direct contact with other species, with the remaining 90% (9/10) allowing indirect contact (adjacent cages or aviaries and shared cleaning, reproductive and feeding utensils).

Considering the studied population individually, 90 birds were housed in couples (n=45 pairs) and 10 were living in communal housing (breeder-9). All included birds from the same breeder were housed in identical conditions. None of the included *P. erithacus* had been previously tested for BFDV.

3.2 PCR detection of circovirus

Amplification of the 12S rDNA housekeeping gene was possible in all samples (*n*=100), reflecting the good quality and integrity of the DNA, as well as the absence of DNA polymerase inhibitors that could lead to false negative results.

The detection of asymptomatic BFDV infection by nested PCR was confirmed in a total of 8 birds, which represents an overall BFVD frequency of 8% (8/100) in this study (Fig. 2).

The BFDV positive birds belonged to a total of six breeders (breeder-1, breeder-4, breeder-6, breeder-7, breeder-8 and breeder-9), corresponding to 60% of the studied collections (Fig. 1, Table 3).

Two breeders (breeder-1 and breeder-4) showed an infection frequency of 20% (2/10 each). Four breeders (breeder-6, breeder-7, breeder-8, and breeder-9) had a frequency of BFDV infection of 10%, corresponding to one positive *P. erithacus* out of the 10 tested per breeder. BFDV infection was not detected in the remaining breeders (breeder-2, breeder-3, breeder-5, and breeder-10) although they had the same management conditions as breeder-6, breeder-7, and breeder-8 (Table 2).

Among the 45 couples tested, 7 had one positive bird, corresponding to 15.6% (7/45) of infected couples. An interesting finding in this study was that the housing partners of the 7 positive birds were all negative for BFDV (Table 3).

Among the 8 positive birds for BFDV, 37.5% (3/8) had contact with soil and faeces, 25% (2/8) were housed indoors, and 12.5% (1/8) had contact with other species. Finally, considering the 92 birds negative for BFDV, 18.5% (17/92) had contact with soil and faeces, 19.6% (18/92) were housed indoors, and 9.8% (9/92) had contact with other species.

No statistically significant differences were found between the housing conditions of infected and non-infected birds ($p > 0.05$).

4. DISCUSSION

In the present study, 8% (8/100) of captive healthy *P. erithacus* were shown to be positive for BFDV, thus being asymptomatic. To our best knowledge, this is the first study aiming to evaluate the presence of BFDV in healthy *P. erithacus* kept in captivity in Portugal. Considering the key role that Portugal seems to have played, in the past, in the dissemination of BFDV (Harkins et al. 2014) and the scarcity of existing data about this virus in Portugal, updated information is essential to better understand its current epidemiology. Furthermore, the importance of this virus in the health and preservation of several Psittacidae species worldwide (Fogell et al. 2016; Bert et al. 2005) makes this subject of the utmost importance.

Despite the existence of discordant data regarding the preferred type of sample to be used, in the present study, blood samples were chosen for the identification of asymptomatic infection (subclinical presentation), as several studies indicate better results with these samples (Fogell et al. 2016; Khalesi 2007). Additionally, the use of blood samples can potentially minimise the occurrence of environmental contamination.

The prevalence of BFDV infection varies worldwide. Comparisons between studies are difficult to establish due to differences related to the study design such as the population studied (symptomatic vs. asymptomatic; wild vs. captive; type of bird management), geographic and temporal variations, and the use of different detection methods, among others.

The overall BFDV frequency of 8% (8/100) found in this study is in agreement with that described in Italy, where a frequency of 7.5% (28/371) was described in blood samples from birds of the genus *Psittacus* kept in captivity. However, these included birds with and without clinical signs of PBFD (Bert et al. 2005). In other European countries, results differ according to the study (Valastanova et al. 2021; Julian et al. 2013; Piçarra 2010; Rahaus and Wolff 2003). In Poland, in captive birds, a frequency of BFDV infection of 17.3% (9/52) was found. The health status of these birds (symptomatic vs. asymptomatic) was not disclosed and blood and/or feather samples were used (Julian et al. 2013). In Germany, a BFDV infection frequency of 39.2%, using feather samples of 146 asymptomatic birds from 32 breeders, was reported. Of these, only 18 belonged to the genus *Psittacus*; however, the authors did

not mention the infection frequency within this genus (Rahaus and Wolff 2003). In the Czech Republic, a BFDV infection frequency of 21.5% (38/117), using feather samples from 117 healthy parrots from 42 facilities, was reported. Of these, 51 belonged to the *P. erithacus* species and 5 were found positive for BFDV infection, accounting for an infection frequency of 10.2% in this species. In this case, nested PCR was used for virus detection (Valastanova et al. 2021). A 2.9% (16/554) BFDV infection frequency was reported in *P. erithacus* kept in captivity in the Barcelona area using blood samples. Interestingly, despite the low BFDV infection frequency reported, this study included birds with and without clinical signs (Piçarra 2010).

Outside of Europe, studies also show varying results and different study designs, thus greatly limiting comparisons between countries (Haddadmarandi et al. 2018; Hakami et al. 2017; Hakimuddin et al. 2016; Huang et al. 2016).

As in other studies, the frequency of asymptomatic BFDV infection found in the present study may be influenced by several factors including the detection methodology chosen. The presence of low viral loads, expected in asymptomatic carriers, could lead to underestimating this frequency, although the use of a nested PCR aimed to minimise this effect. Sequencing of the entire virus genome in future studies should be pursued, as it would allow the study of the BFDV variants circulating in Portugal.

Information about the housing and management conditions of the birds included in this study was registered to identify practices and handling conditions that could be considered risk factors for the dissemination of BFDV. This study showed that, despite the importance of BFDV for captive and wildlife birds, several breeders kept favourable conditions for virus maintenance. For example, contact between birds, even if indirectly through fomites, may be a risk factor for environmental contamination and BFDV dissemination (Bert et al. 2005). One could hypothesise that this cross-contamination is particularly important in breeders with a high entry and exit of birds of different species from the bird collections, as was the case in this study. Also, direct contact with soil and faeces, which was allowed in birds from two breeders, is one of the most common routes of virus dissemination, since BFDV is excreted in faeces, feathers, and shedding dust (Regnard et al. 2017; Araújo 2011; Phalen 2006). Since eradication of BFDV is unlikely, implementation of disinfection measures with known effective impact on this non-enveloped virus should be considered (Amery-Gale et al. 2017).

Another important finding from this study was the lack of systematic BFDV testing of birds before its introduction in the bird collections. Screening for infection is seen as an additional cost by most breeders that only test birds when they evidence suspected clinical signs. Also, the absence of effective treatment (Tomasek et al. 2008) likely leads breeders to underestimate the importance of BFDV testing, putting their bird collections at a higher risk. This misperception can have consequences, both economically and for the captive and wild birds' health. Increasing breeders' knowledge of how the virus is spread may help to implement preventive measures to stop BFDV from entering and spreading within bird collections.

Considering that BFDV has a high transmission rate, it was surprising to find BFDV-positive and -negative birds living together. Several explanations could be hypothesised. These BFDV-negative birds could have higher levels of BFDV antibodies and lower viral loads, hindering virus detection. Also, since only healthy birds were included, it may be possible that these BFDV-negative birds were recently infected or were in a transitory infection process (Khalesi 2007; Phalen 2006).

BFDV-positive asymptomatic birds are silent carriers that can spread the virus and threaten entire collections of birds, many of which include endangered species. Moreover, the export of infected captive birds to countries with endemic and susceptible Psittaciformes may increase the risk of extinction of endangered birds worldwide.

Future longitudinal studies on asymptomatic *P. erithacus* would be of great value to better understand the virus shedding over time and the transmission dynamics in captivity, as well as to estimate the true role of these birds as BFDV carriers.

Also, since the occurrence of seasonal fluctuation in the frequency of BFDV in birds has been proven (Martens et al.2020c), it would be of great interest to study this variation in captive *P. erithacus* housed in the exterior.

In Portugal, as in other European countries, there are no endemic birds of the Psittacidae family; however, an increase in the number of invasive Psittacidae species is emerging (Alonso et al. 2022). Furthermore, studies have shown that BFDV can also be detected in non-psittacine birds (Ahaduzzaman et al. 2022; Amery-Gale et al. 2017). In Australia, a high frequency of BFDV (20.0%, 21/105) was observed in liver samples from captive and wild asymptomatic non-psittacine species (Amery-Gale et al. 2017). In Bangladesh, another study reported a

frequency of 40% (4/10) BFDV infection in non-psittacine species (Ahaduzzaman et al. 2022). To the best of our knowledge, BFDV infection in wild invasive Psittaciformes or non-psittacine birds has not been reported in Portugal so far. Still, it is reasonable to assume that these invasive Psittacidae species may become infected and, eventually, contribute to a greater spread of this virus. Thus, future BFDV infection monitoring in these populations is essential.

Table 1: Primers used in this study

Primers	**Target**	**Sequences**	**Size**	**Reference**
12S rDNA L 12S rDNA H	12S rDNA	GGA TTA GAT ACC CCA CTA TGC AGG GTG ACG GGC GGT ATG TAC G	436bp	(Bert et al. 2005)
PBFD 2 PBFD 4	ORF V1[1]	AAC CCT ACA GAC GGC GAG GGT CAC AGT CCT CCT TGT ACC	718bp	(Tomasek et al. 2008)
PBFD 251 PBFD 609	ORF V1[2]	ACT TAC CCT GGG CAT TGT GGC G GGC GGA GCA TCT CGC AAT AAG G	359bp	(Tomasek et al. 2008)

[1]Between nucleotides 182 and 899 of the BFDV genome;
[2]Between nucleotides 251 and 609 of the BFDV genome.

Table 2 Housing conditions of birds per breeder.

Breeder ID	Contact with soil and faeces		Environment		Housing		Direct contact with other species	
	Yes	No	Interior[1]	Exterior[2]	Couple[3]	Communal[4]	Yes	No
1	X			X	X			X
2		X		X	X			X
3		X		X	X			X
4		X	X		X			X
5		X	X		X			X
6		X		X	X			X
7		X		X	X			X
8		X		X	X			X
9	X			X		X	X	
10		X		X	X			X

[1]Bird cages were kept indoors, thus having poor ventilation; [2]Bird cages were kept outdoors; [3]Two birds per cage, namely one *P. erithacus* female and one male; [4]Several birds of different species in the same aviary, including five *P. erithacus* females and five males.

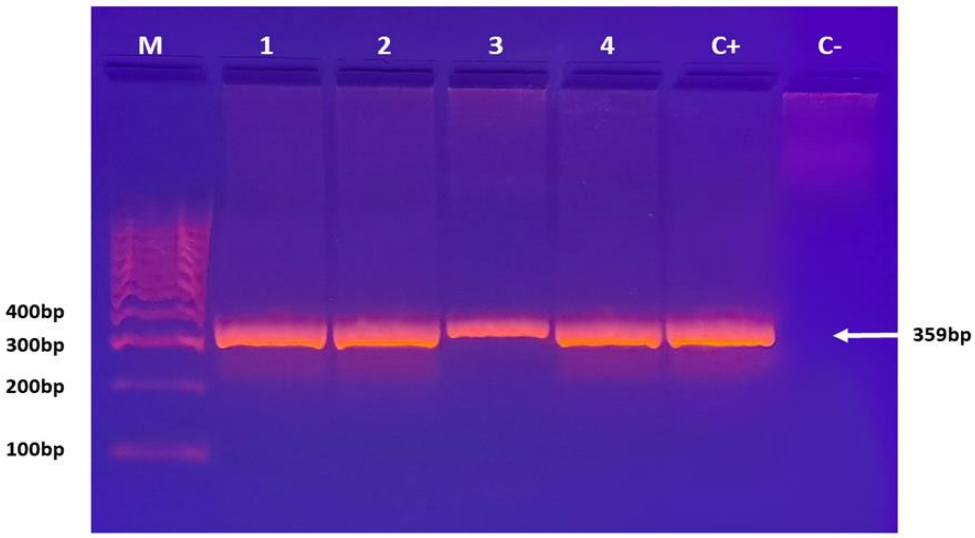

Fig. 2 BFDV amplification results were observed after a 2% (w/v) agarose gel electrophoresis of the nested PCR products. M: Hypperladder 100 bp molecular weight marker (Bioline®, London UK). 1 to 4: PCR product from four BFDV positive samples. C+: Positive control. C-: Negative control.

Table 3 BFDV positive *P. erithacus.*

Breeder ID	**Couple ID**	**Bird ID**	**PCR result**
1	1	PE0101 PE0102	Negative Positive
	2	PE0103 PE0104	Positive Negative
4	18	PE0405 PE0406	Positive Negative
	19	PE0407 PE0408	Positive Negative
6	30	PE0609 PE0610	Negative Positive
7	34	PE0707 PE0708	Positive Negative
8	40	PE0809 PE0810	Negative Positive
9	Communal housing	PE0908 PE0901-07, 09, 10	Positive Negative

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Gonçalo Portela, Mário Nóbrega, Tiago Santos, Cátia Marques, and Margarida Alves. The first draft of the manuscript was written by Gonçalo Portela and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding: This research was funded by Lusófona University (projecto FMV_Estágios_21-22).

Ethics approval: The animal study protocol was approved by the Institutional Ethics Committee of Lusófona University (parecer 4-2022).

Consent to participate: Not applicable to this article.

Consent to publish: Not applicable to this article.

Data Availability: Data sharing does not apply to this article.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

5. REFERENCES

Ahaduzzaman, M, Nath C, Hossain, MS (2022) Evidence of circulation of beak and feather disease virus in captive psittacine and non-psittacine birds in Bangladesh. Archives of Virology. https://doi.org/10.1007/s00705-022-05579-4

Allgayer MC, Pereira RA (2014) Doenças Virais em Psittaciformes. In: Silva JCR Tratado de animais selvagens-medicina veterinária, 2nd edn. Editora Roca, pp 1339-1341

Alonso H, Andrade J, Teodósio J, Lopes A (2022). O estado das aves em Portugal, 2022. Sociedade Portuguesa para o Estudo das Aves, Lisboa. https://spea.pt/wp-content/uploads/2022/11/Estado-das-Aves_PT_2022.pdf. Accessed 8 March 2023

Amery-Gale J, Marenda MS, Owens J, Eden PA, Browning GF, Devlin JM (2017) A high prevalence of beak and feather disease virus in non-psittacine Australian birds. Journal of Medical Microbiology. https://doi.org/10.1099/jmm.0.000516

Araújo AV (2011) Doença do bico e das penas: avaliação em psitacídeos nativos apreendidos em Minas Gerais. Dissertation, Universidade Federal de Minas Gerais

Bert E, Tomassone L, Peccati C, Navarrete MG, Sola SC (2005) Detection of beak and feather disease virus (BFDV) and avian polyomavirus (APV) DNA in psittacine birds in Italy. Journal of Veterinary Medicine. https://doi.org/10.1111/j.1439-0450.2005.00823.x

Das S, Sarker S, Forwood JK, Ghorashi SA, Raidal SR (2014) Characterization of the whole-genome sequence of a beak and feather disease virus isolate from a Mallee Ringneck parrot (Barnardius zonarius barnardi). Genome Announcements. https://doi.org/10.1128/genomea.00708-14

Das S, Smith K, Sarker S, Peters A, Adriaanse K, Eden P, Raidal SR et al (2020) Repeat spillover of beak and feather disease virus into an endangered parrot highlights the risk associated with endemic pathogen loss in endangered species. Journal of Wildlife Diseases. https://doi.org/10.7589/2018-06-154

Desingu PA, Nagarajan K (2022) Detection of beak and feather disease virus in India and its implications. Transboundary and Emerging Diseases. https://doi.org/10.1111/tbed.14749

Fogell, DJ, Martin RO, Groombridge JJ (2016) Beak and feather disease virus in wild and captive parrots: an analysis of geographic and taxonomic distribution and methodological trends. Archives of virology. https://doi.org/10.1007/s00705-016-2871-2

Gerlach H (1994) Viruses. In: Ritchie BW, Harrison GJ, Harrison LR (ed) Avian Medicine: Principles and Application. Lake Worth: Wingers Publishing, pp. 862-948

Greenacre CB (2005) Viral diseases of companion birds. Veterinary Clinics: Exotic Animal Practice. https://doi.org/10.1016/j.cvex.2004.09.005

Haddadmarandi MR, Madani SA, Nili H, Ghorbani A (2018) Molecular detection and characterization of beak and feather disease virus in psittacine birds in Tehran, Iran. Iranian journal of veterinary research. https://pubmed.ncbi.nlm.nih.gov/29805458/

Hakami A, Al-Ankari A, Zaki M, Yousif A (2017) Isolation and characterization of psittacine beak and feather disease virus in Saudi Arabia using molecular technique. International Journal of Avian & Wildlife Biology. https://doi.org/10.15406/ijawb.2017.02.00010

Hakimuddin F, Abidi F, Jafer O, Li C, Wernery U, Hebel C, Khazanehdari K (2016) Incidence and detection of beak and feather disease virus in psittacine birds in the UAE. Biomolecular detection and quantification. https://doi.org/10.1016/j.bdq.2015.10.001

Harkins GW, Martin DP, Christoffels A, Varsani A (2014) Towards inferring the global movement of beak and feather disease virus. Virology. https://doi.org/10.1016/j.virol.2013.11.033

Huang SW, Chiang YC, Chin CY, Tang PC, Liu PC, Wang CY (2016) The phylogenetic and recombinational analysis of beak and feather disease virus Taiwan isolates. Archives of virology. https://doi.org/10.1007/s00705-016-2963-z

Jackson B, Harvey C, Galbraith J, Robertson M, Warren K, Holyoake C, Varsani A et al (2014) Clinical beak and feather disease virus infection in wild juvenile eastern rosellas of New Zealand; biosecurity implications for wildlife care facilities. New Zealand Veterinary Journal. https://doi.org/10.1080/00480169.2014.909750

Julian L, Piasecki T, Chrząstek K, Walters M, Muhire B, Harkins GW, Martin DP, Varsani A (2013) Extensive recombination detected amongst Beak and feather disease virus isolates from breeding facilities in Poland. Journal of General Virology. https://doi.org/10.1099/vir.0.050179-0

Kessler S, Heenemann K, Krause T, Twietmeyer S, Fuchs J, Lierz M, Rubbenstroth D et al (2020) Monitoring of free-ranging and captive Psittacula populations in Western Europe for avian bornaviruses, circoviruses and polyomaviruses. Avian Pathology.. https://doi.org/10.1080/03079457.2019.1681359

Khalesi B (2007) Studies of beak and feather disease virus infection. Dissertation, Murdoch University

Ma J, Tian Y, Zhang M, Wang W, Li Y, Tian F, Sun J et al (2019) Identification and characterization of novel genotypes of psittacine beak and feather disease virus from budgerigar in China. Transboundary and emerging diseases. https://doi.org/10.1111/tbed.13274

MacLachlan NJ, Dubovi EJ (2017) Circoviridae and Anelloviridae. In: Fenner F (ed) Fenner's Veterinary Virology, 5th edn. Elsevier, London, pp 259-268

Martens JM, Stokes HS, Berg ML, Walder K, Bennett AT (2020c) Seasonal fluctuation of beak and feather disease virus (BFDV) infection in wild Crimson Rosellas

(*Platycercus elegans*). Scientific reports. https://doi.org/10.1038/s41598-020-64631-y

Martens JM, Stokes HS, Berg ML, Walder K, Raidal SR, Magrath MJ, Bennett AT (2020a) Beak and feather disease virus (BFDV) prevalence, load and excretion in seven species of wild caught common Australian parrots. PloS one. https://doi.org/10.1371/journal.pone.0235406

Martens JM, Stokes HS, Berg ML, Walder K, Raidal SR, Magrath MJ, Bennett AT (2020b) A non-invasive method to assess environmental contamination with avian pathogens: beak and feather disease virus (BFDV) detection in nest boxes. PeerJ. http://dx.doi.org/10.7717/peerj.9211

Massaro M, Ortiz-Catedral L, Julian L, Galbraith JA, Kurenbach B, Kearvell J, Kemp J, van Hal J, Elkington S, Taylor G, Greene T et al (2012) Molecular characterisation of beak and feather disease virus (BFDV) in New Zealand and its implications for managing an infectious disease. Archives of Virology. https://doi.org/10.1007/s00705-012-1336-5

Ortiz-Catedral L, Wallace CJ, Heinsohn R, Krebs EA, Langmore NE, Vukelic D, Masello JF et al (2022) A PCR-based retrospective study for beak and feather disease virus (BFDV) in five wild populations of parrots from Australia, Argentina and New Zealand. Diversity. https://doi.org/10.3390/d14020148

Pendl H, Tizard I (2016) Immunology. In: Speer BL (Ed.) Current Therapy In Avian Medicine And Surgery, 1st edn. Elsevier Saunders Inc, Missouri, pp 400-432

Peters A, Patterson EI, Baker BG, Holdsworth M, Sarker S, Ghorashi SA, Raidal SR (2014) Evidence of psittacine beak and feather disease virus spillover into wild critically endangered orange-bellied parrots (Neophema chrysogaster). Journal of Wildlife Diseases. https://doi.org/10.7589/2013-05-121

Phalen DN (2006) Implications of viruses in clinical disorders. In Harrison GJ, Lightfoot TL (Ed) Clinical Avian Medicine. Spix Publishing, Palm Beach

Philadelpho NA, Chacón RD, Diaz Forero AJ, Guimarães MB, Astolfi-Ferreira CS, Piantino Ferreira AJ (2022) Detection of aves polyomavirus 1 (APyV) and beak and feather disease virus (BFDV) in exotic and native Brazilian Psittaciformes. Brazilian Journal of Microbiology. https://doi.org/10.1007/s42770-022-00785-3

Piçarra JPDSC (2010) Estudo sobre a detecção do circovírus aviário psitacídeos domésticos na região de Barcelona–Espanha. Dissertation, Universidade Técnica de Lisboa

Portas T, Jackson B, Das S, Shamsi S, Raidal SR (2017) Beak and feather disease virus carriage by Knemidocoptes pilae in a sulphur crested cockatoo (Cacatua galerita) with mange, concurrent cestodiasis and deep mycotic encephalitis. Australian Veterinary Journal. https://doi.org/10.1111/avj.12649

Pyne M, Sanctuary CW, Currumbin GC (2005) Psittacine beak and feather disease. In Proceedings of the National Wildlife Rehabilitation Conference. https://www.awrc.org.au/uploads/5/8/6/6/5866843/awrc_michael_pyne.pdf. Accessed 8 March 2023

Rahaus M, Wolff MH (2003) Psittacine beak and feather disease: a first survey of the distribution of beak and feather disease virus inside the population of captive psittacine birds in Germany. Journal of Veterinary Medicine. https://doi.org/10.1046/j.1439-0450.2003.00696.x

Rahaus M, Desloges N, Probst S, Loebbert B, Lantermann W, Wolff MH (2008) Detection of beak and feather disease virus DNA in embryonated eggs of psittacine birds. Veterinarni Medicina 53, 53-58.

Raidal SR, Peters A (2018) Psittacine beak and feather disease: Ecology and implications for conservation. Emu – Austral Ornithology. https://doi.org/10.1080/01584197.2017.1387029

Raue R, Johne R, Crosta L, Burkle M, Gerlach H, Muller H (2004) Nucleotide sequence analysis of a C1 gene fragment of psittacine beak and feather disease virus amplified by real-time polymerase chain reaction indicates a possible existence of genotypes. Avian Pathology. https://doi.org/10.1080/03079450310001636219

Regnard GL, Rybicki EP, Hitzeroth II (2017) Recombinant expression of beak and feather disease virus capsid protein and assembly of virus-like particles in Nicotiana benthamiana. Virology journal. https://doi.org/10.1186/s12985-017-0847-9

Sarker S, Das S, Ghorashi SA, Forwood JK, Raidal SR (2014) Molecular characterization of genome sequences of beak and feather disease virus from the Australian twenty-eight parrot (Barnardius zonarius semitorquatus). Genome announcements. https://doi.org/10.1128/genomea.01255-14

Tomasek O, Kubicek O, Tukac V (2008) Comparison of three template preparation methods for routine detection of beak and feather disease virus and avian polyomavirus with single and nested polymerase chain reaction in clinical specimens. Avian Pathology. https://doi.org/10.1080/03079450801902047

Valastanova M, Petrikova M, Kulikova L, Knotek Z (2021) Psittacine beak and feather disease virus and avian polyomavirus detection rate in clinically healthy captive birds in the Czech Republic. Veterinární medicína. https://doi.org/10.17221/22/2020-VETMED