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A high prevalence of beak and feather disease virus in non-psittacine Australian birds

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Abstract:	Purpose: Beak and feather disease virus (BFDV) is a circovirus and the cause of psittacine beak and feather disease (PBFD). This disease is characterised by feather and beak deformities and is a recognised threat to endangered Psittaciformes (parrots and cockatoos). The role that non-psittacine birds may play as reservoirs of infection is unclear. This study aimed to begin addressing this gap in our knowledge of PBFD. Methodology: Liver samples were collected from birds presented to the Australian Wildlife Health Centre at Zoos Victoria's Healesville Sanctuary for veterinary care between December 2014 and December 2015, and tested for BFDV DNA using PCR coupled with sequencing and phylogenetic analyses. Results/Key findings: Overall BFDV was detected in 38.1% of 210 birds. BFDV was detected at high prevalence (56.2%) in psittacine birds, in the majority of cases without any observed clinical signs of PBFD. We also found that BFDV was more common in non-psittacine species than previously recognised, with BFDV detected at 20.0% prevalence in the non-psittacine birds tested, including species with no clear ecological association with psittacines, and without showing any detectable clinical signs of BFDV infection. Conclusion: Further research to determine the infectivity and transmissibility of BFDV in non-psittacine species is indicated. Until such work is undertaken the findings from this study suggest that every bird should be considered a potential carrier of BFDV, regardless of species and clinical presentation. Veterinary clinics and wildlife rehabilitation facilities caring for birds that are susceptible to PBFD should reconsider biosecurity protocols aimed at controlling BFDV.

A high prevalence of beak and feather disease virus in non-psittacine Australian birds

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Abbreviations: AWHC = Australian Wildlife Health Centre; BFDV = beak and feather disease virus; BLAST® = Basic Local Alignment Search Tool; BLASTN® = Nucleotide Basic Local

Alignment Search Tool; 95% C. I. = 95% confidence interval; GTR = general-time-reversible;

PBFD = psittacine beak and feather disease

Abstract

Purpose: Beak and feather disease virus (BFDV) is a circovirus and the cause of psittacine beak and feather disease (PBFD). This disease is characterised by feather and beak deformities and is a recognised threat to endangered Psittaciformes (parrots and cockatoos). The role that non-psittacine birds may play as reservoirs of infection is unclear. This study aimed to begin addressing this gap in our knowledge of PBFD.

Methodology: Liver samples were collected from birds presented to the Australian Wildlife Health Centre at Zoos Victoria's Healesville Sanctuary for veterinary care between December 2014 and December 2015, and tested for BFDV DNA using PCR coupled with sequencing and phylogenetic analyses.

Results/Key findings: Overall BFDV was detected in 38.1% of 210 birds. BFDV was detected at high prevalence (56.2%) in psittacine birds, in the majority of cases without any observed clinical signs of PBFD. We also found that BFDV was more common in non-psittacine species than previously recognised, with BFDV detected at 20.0% prevalence in the non-psittacine birds tested, including species with no clear ecological association with psittacines, and without showing any detectable clinical signs of BFDV infection.

Conclusion: Further research to determine the infectivity and transmissibility of BFDV in non-psittacine species is indicated. Until such work is undertaken the findings from this study suggest that every bird should be considered a potential carrier of BFDV, regardless of species and clinical presentation. Veterinary clinics and wildlife rehabilitation facilities caring for birds that are susceptible to PBFD should reconsider biosecurity protocols aimed at controlling BFDV.

Introduction

Beak and feather disease virus (BFDV), the circoviral aetiological agent of psittacine beak and feather disease (PBFD), is the most common viral pathogen of both captive and wild Psittaciformes in its native Australia [1-6]. Infection with BFDV is endemic in Australia, with wild psittacine populations across the continent carrying a rich viral genetic diversity [2, 6]. Infection with BFDV can cause severe feather and beak deformities and is potentially fatal, being an important cause of morbidity and mortality in wild and captive psittacine birds in Australia and in other countries [1-9]. Beak and feather disease virus is a small, non-enveloped virus with an icosahedral capsid that belongs to the genus Circovirus within the family Circoviridae [1, 10-15]. Circoviruses are the smallest and simplest autonomously replicating pathogens known to infect vertebrates [1, 6, 11, 12]. Psittacine birds are the natural host of BFDV [16], and while the virus is thought to be capable of infecting all psittacines, different species vary in their susceptibility to infection and development of disease [6, 9, 11, 12]. There is currently no treatment or vaccine available for PBFD.

BFDV is a pathogenic host-generalist capable of flexible host-switching amongst psittacid avifauna, with Australian BFDV genetic clades representing a diverse host species mosaic [2]. BFDV occupies an entangled multispecies ecological niche, but Das *et al.* suggested that the high degree of contemporary host-switching amongst all available host lineages can be explained by deep and ancient intra-lineage host phylogeny [11]. BFDV had its origins in and has co-evolved with psittacine birds [2]. Even though neotropical, African and New Zealand parrots are susceptible to BFDV infection, there is strong phylogeographic genetic evidence of a post-Gondwanan origin of the ancestral virus in psittacine birds in Australia, with anthropogenic global spread of BFDV to a wide variety of free-ranging and captive psittacines over the past 150 years as a result of the live pet bird trade [2, 6, 9, 17].

Psittacine circoviral disease affecting endangered psittacine species was listed as a key threatening process by the Australian Government in 2001 under the Environment Protection and Biodiversity Conservation Act 1999 [2, 15, 18]. A number of critical knowledge gaps have been identified that need to be addressed in order to better inform management strategies for this key threat, including the significance of cross-species transmission, the potential for other species to act as reservoirs of the virus and the extent of environmental contamination [19]. This study aimed to undertake surveillance of both psittacine and non-psittacine avian species in order to address some of these knowledge gaps, by determining the prevalence of BFDV infection in wild and captive Victorian birds and examining the phylogenetic relationships between any BFDV haplotypes that were detected.

Methods

Liver samples were collected at post-mortem from 210 birds presented to the Australian Wildlife Health Centre (AWHC) at Zoos Victoria's Healesville Sanctuary for veterinary diagnosis and treatment between December 2014 and December 2015. All birds had either died or were euthanised for reasons unrelated to this study. The location, date of collection, species and any other available and relevant details about the bird's history, including the reason for its admission, were collected by AWHC staff. A sample of liver was collected into a sterile tube using aseptic technique at necropsy (with autoclaved instruments) and stored at -20 °C. Samples of liver tissue were crushed with a sterile swab and the swab placed into 500 μL RNAlater (Ambion), then vortexed for 10 seconds before taking 200 μL aliquots from which nucleic acid was extracted using the VX Universal Liquid Sample DNA Extraction Kit (Qiagen) and a Corbett X-tractor Gene Robot (Corbett Robotics), according to the manufacturer's instructions.

Extracted DNA was tested for the presence of BFDV by PCR as described previously [13]. Oligonucleotide primers specifically targeting the capsid protein encoding region (ORF C1) of the

BFDV genome (forward, BFDV-F, 5'-GGGTCCTCCTTGTAGTGGGATC-3', and reverse, BFDV-R, 5'-CAGACGCCGTTTCACAACCAATAG-3') were used for PCR amplification of an approximately 495 bp fragment [13]. Diethyl pyrocarbonate-treated (DEPC) water was used as template in negative control reactions. Each 25 µL reaction mixture contained 5 µL 5× Green GoTaq® Flexi Buffer (Promega), 2 µM of each primer, 2.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 1.25 U GoTaq® Flexi DNA Polymerase (Promega), 5 µL extracted DNA template and DEPC water to volume. PCR amplification was performed by incubation through initial denaturation at 94 °C for 3 minutes, then 40 cycles of 94 °C for 20 seconds (denaturation), 63 °C for 20 seconds (annealing) and 72 °C for 30 seconds (extension), then a final extension at 72 °C for 3 minutes. Products amplified by PCR were visualised by UV transillumination after electrophoresis through a 1.5% agarose gel containing SYBR™ Safe DNA Gel Stain (Invitrogen) in 0.5× TBE buffer. HyperLadder™ 100 bp (Bioline) DNA size markers were used to estimate amplicon size.

For all faint bands and PCR products obtained from non-psittacine birds, the original liver sample was re-swabbed and nucleic acid freshly extracted manually with a QIAamp Viral RNA Mini Kit (Qiagen) using the spin protocol according to manufacturer's instructions, before PCR was repeated to verify results using the freshly extracted DNA template.

PCR products were purified from PCR reaction mixtures using the QIAquick® Gel Extraction Kit Microcentrifuge Protocol (Qiagen). Selected amplicons were directly sequenced using BigDye® Terminator version 3.1 chemistry (Applied Biosystems). Sequencing analysis focused on PCR products that produced strong bands following gel electrophoresis. Samples selected for sequencing were also chosen to represent PCR products amplified from both psittacine and non-psittacine hosts. Nucleotide sequences were compared with publicly available sequences in the GenBank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/genbank/)

using the NCBI Nucleotide Basic Local Alignment Search Tool (BLASTN) online algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A global alignment of partial ORF C1 sequences (produced using Geneious [Biomatters]) was used to generate a PhyML maximum likelihood phylogenetic tree using the general-time-reversible (GTR) nucleotide substitution model with four substitution rate categories [20]. The reliability of each tree branch was calculated using 1000 replicates in a bootstrap resampling analysis. The 95% confidence intervals (95% C. I.) for sample proportions and prevalence estimates were calculated using the Jeffreys method [21].

Results

Detection of BFDV in captive and wild birds presented to the AWHC at Healesville Sanctuary

DNA extracted from liver samples from 192 wild and 18 captive birds was screened for the presence of BFDV DNA by PCR, with BFDV DNA being detected in 80 of these 210 birds (38.1% overall prevalence across all birds sampled at necropsy). BFDV DNA was detected in 59 of the 105 psittacine birds tested (56.2% prevalence) – that is, in 67.7% of the 31 cacatuids and 51.4% of the 74 psittacids tested (see Table 1). BFDV DNA was also unexpectedly detected in 21 of the 105 non-psittacine birds tested (20.0% prevalence). These results are shown in Table 1, along with the 95% confidence interval values for BFDV prevalence in the differerent populations examined in this study. No observable clinical signs of PBFD were noted in any of the non-psittacine birds in which BFDV was detected (see Table S1).

For samples that produced faint bands, or results that could not be clearly interpreted, when initially tested by PCR, the DNA was re-extracted and used as template in a second PCR. Of these 37 samples, 21 were confirmed positive when re-tested, nine samples that produced faint bands when initially tested did not produce any bands when tested for a second time, and seven samples that were tentatively classified as negative when initially tested (but showed some possible non-specific amplification of DNA on gel electrophoresis) where confirmed to be negative. Samples that tested

weakly positive initially, but not when re-tested, were classified as negative. No PCR products were amplified from any of the negative control reactions.

Molecular characterisation of BFDV detected in captive and wild birds

Sequence information was obtained for 39 of the 80 PCR products that were obtained in this study. Of these 39 products, 31 yielded high quality sequence suitable for further analysis. A summary of the BLAST analysis of these 31 sequences, along with their source and any reported clinical signs of PBFD are shown in Table S1. Fig. 1 shows a phylogenetic analysis of these 31 sequences. There was no strong consistent pattern of associations between infected host species and viral genotypes (consistent wih a pathogen capable of flexible host-switching), however some clustering of sequences was observed. In general, groups of BFDV sequences obtained in this study clustered together, often alongside published BFDV sequences also obtained from Victorian birds (see Fig. 1 and Table S1). With the exception of two sequences obtained from captive Scaly-breasted lorikeets (*Trichoglossus chlorolepidotus*), which clustered with published BFDV sequences from cacatuid hosts, all BFDV sequences from lorikeets clustered together, along with two sequences from Australian magpies. Other BFDV sequences obtained from members of the superfamily Corvoidea clustered with BFDV sequences from cacatuid hosts, as did BFDV sequences obtained from the Tawny frogmouths, Australian white ibis, Powerful owl and captive Orange-bellied parrot in the present study. BFDV sequences obtained from rosellas in our study consistently clustered with previously published BFDV sequences from rosellas, together with the sequences obtained from a captive Australian king parrot (*Alisterus scapularis*) and the Brown goshawk in the present study (Fig. 1).

Discussion

BFDV is a widespread and highly prevalent multi-host pathogen recognised as a serious conservation threat to small, isolated or naïve wild psittacine populations globally. It has been

implicated as a cause of wild parrot declines in Australia and Mauritius [9, 11, 22]. Host-switching occurs at a high rate across divergent Psittaciformes, with rare spillovers into distantly related non-psittacine bird species also reported [6, 10, 11, 16]. The emerging evidence suggests that all species within the order Psittaciformes may be susceptible to infection by all genotypes of BFDV [2, 4]. Thus all BFDV variants may threaten wild psittacine populations [17]. Although BFDV occurs naturally in Australia and is commonly detected in both wild and captive populations of Australian psittacines, it has the potential to cause catastrophic losses where populations are already low and genetic diversity is reduced [7].

BFDV is copiously shed in faeces and feather dander from infected birds [16], with feather dust from PBFD-affected birds containing as many as one billion virus particles per microlitre [1]. Transmission occurs by direct contact, ingestion, inhalation of contaminated aerosols or via infected fomites [1, 13]. Psittacine birds commonly live in flocks and nest in tree hollows, which favours transmission of the virus within a population [1]. The virus may remain viable in tree hollows and other nesting sites for many years [8]. Nest hollows are likely to be the critical site for natural transmission of BFDV through contact with faeces and feather dust, and as cockatoos, parrots and lorikeets all compete closely for nest hollows, there is ample opportunity for sharing of different viral genotypes [11]. Numerous BFDV genome variants may be present in individual infected birds, resulting in a population of multiple genetic variants within an infective dose [16, 23]. During an outbreak of PBFD in critically endangered Orange-bellied parrots (*Neophema chrysogaster*), at least 13 genotypic variants were identified in four different birds, with one individual containing up to seven genetic variants [24]. In the present study the BFDV sequences obtained from at least two birds provided preliminary evidence of such co-infection, but this would need to be confirmed using cloning, followed by DNA sequencing, and for ease of analysis these sequences were excluded from both the BLAST searches and phylogenetic comparisons.

Of the samples from non-psittacine birds in the present study, 19 of the 21 samples that are reported as positive in this report tested positive when tested initially, and also tested positive when DNA was freshly extracted and re-tested. Only two positive samples from non-psittacine birds were not re-tested, as the bands from the initial extraction and PCR were very strong. The inability to re-amplify product from nine samples that produced faint bands when first tested may be consistent with degradation of DNA during sample storage. These nine samples included samples from both psittacine (two samples) and non-psittacine birds (seven samples).

Whether non-psittacine species can carry and disseminate infection has been identified as a critical gap in our knowledge about BFDV [19]. Until recently BFDV infection was thought to be specific and restricted to the order Psittaciformes [6, 10, 16, 25], but there is growing evidence that distantly related non-psittacine orders, including Coraciiformes and Strigiformes, can be naturally infected with BFDV from parrots [6, 11, 16]. As psittacines are abundant and widely distributed in Australia and there is high prevalence of BFDV infection in many of the most common psittacine species, birds from other orders would frequently be exposed to BFDV [16].

The first report of BFDV infection and disease in a non-psittacine avian host was in a captive flock of Gouldian finches (*Erythrura gouldiae*) in Italy that had feather lesions, feather loss and beak disorders suggestive of PBFD, with icosahedral non-enveloped virions morphologically similar to circovirus particles seen in ultramicrographs of feather quill homogenates [10]. A decade earlier BFDV DNA had been detected in feather samples from four clinically normal captive Hill mynas (*Gracula religiosa*) in Germany by PCR [25]. BFDV infection has also been detected in Rainbow bee-eaters (*Merops ornatus*), members of the order Coraciiformes, collected from the wild in central Australia in 2014 [16]. The affected juvenile Rainbow bee-eaters appeared to be transiently infected and developed plumage defects within a few weeks of being captured [16]. The BFDV genome had the highest similarity (95.6% pairwise nucleotide identity) to the BFDV from a wild

Red-tailed black cockatoo (*Calyptorhynchus banksii*) [16], and it was concluded that infection most likely occurred before the birds were collected [16]. Rainbow bee-eaters are predominantly insectivorous migratory birds and burrow new nesting tunnels into the sand of river beds each year [16]. Thus they do not intimately share any ecological niche with psittacine species, but the chicks may have been infected within their nest burrows, or via ingestion of BFDV-contaminated insect vectors, such as hippoboscid flies, which are common ectoparasites of psittacines, or via other flying haematophagous insects that cohabit parrot nesting hollows [16]. Whilst ingestion of insect vectors has not yet been proven as a route of transmission for BFDV, the same may be true for the insectivorous non-psittacine birds in which BFDV was detected in the present study. All of the non-psittacine species in which BFDV DNA was detected in the present study are known to eat insects [26].

In the present study BFDV DNA was detected in four of the 13 wild Laughing kookaburras (*Dacelo novaeguineae*) and the single wild Sacred kingfisher (*Todiramphus sanctus*) tested, all members of the same order as Rainbow bee-eaters. The majority of Laughing kookaburra nests are in tree hollows [27, 28], and they feed on a wide array of invertebrate and vertebrate prey [29]. The Sacred kingfisher is a seasonal latitudinal migrant with nesting usually occurring in tree hollows or termite mounds [29], and their diet includes a diverse range of invertebrate and small vertebrate prey [29]. Thus the most likely source of acquisition of BFDV for Laughing kookaburras and Sacred kingfishers would be nesting in a tree hollow previously occupied by psittacine birds, or possibly by ingestion of BFDV-contaminated insect vectors or BFDV-infected psittacine birds.

In the present study BFDV DNA was detected in five of 23 wild Tawny frogmouths (*Podargus strigoides*) tested. BFDV sequences obtained from all five of these birds had highest nucleotide identity with published BFDV sequences from cacatuid hosts. The Tawny frogmouth is an insectivorous nocturnal ground-feeder [30], so its most likely sources of BFDV would be

environmental exposure or ingestion of BFDV-contaminated insect vectors. Ingestion of insect vectors is also a possible source of BFDV for the captive Hardhead duck (*Aythya australis*) and wild Australian white ibis (*Threskiornis moluccus*) in which BFDV DNA was detected in the present study, as is general exposure to a BFDV-contaminated environment – which is true for all birds in Australia, where psittacine birds are abundant across the continent and BFDV infection is highly prevalent amongst their populations [16].

In 2015 BFDV infection was identified in a dead fledgling Powerful owl (*Ninox strenua*) in Sydney, New South Wales, with feather lesions consistent with PBFD [6]. The BFDV genome from this bird clustered with BFDV genotypes obtained from Rainbow lorikeets, known prey of Powerful owls, suggesting that ingestion of a BFDV-infected lorikeet or nesting in a tree hollow previously occupied by a lorikeet family were the most likely possible sources of this cross-order host-switch event [6]. The BFDV sequence obtained from a Powerful owl in the present study (in which Columbid herpesvirus 1 was also detected, results not shown) clustered with BFDV genotypes obtained from cockatoos, another significant component of the Powerful owl's diet. Furthermore cockatoos, parrots, lorikeets and Powerful owls all compete for the same nesting hollows [6]. Thus it is possible that this Powerful owl became infected either in a tree hollow previously occupied by psittacine birds, or by ingestion of a BFDV-infected psittacine bird. No feather lesions were noted on clinical or post-mortem examination of this Powerful owl, but it is possible that immunosuppression caused by BFDV infection predisposed it to its fatal Columbid herpesvirus 1 infection.

In the present study BFDV DNA was detected from an additional three birds of prey: a Southern boobook (*Ninox boobook*), a Barn owl (*Tyto alba*) and a Brown goshawk (*Accipiter fasciatus*). Thus BFDV DNA was detected in 36.4% of the 11 wild birds of prey tested. Southern boobooks are obligate tree hollow-nesting birds [28, 31], and their diet is composed of invertebrate and vertebrate

283 prey [32]. Likewise the diet of the Barn owl in Victoria consists mainly of small mammals,
284 although they have also been recorded preying on small birds, insects, lizards and frogs, and their
285 nest is usually a tree hollow [33]. Brown goshawks construct stick nests high in mature *Eucalyptus*
286 trees [34]. Birds made up 63% of the prey items of the Brown goshawk in a study in Victoria, with
287 mammals, reptiles, insects and crustaceans comprising the remainder of their diet [35]. It is possible
288 that these birds of prey acquired BFDV either in a nesting hollow previously occupied by psittacine
289 birds, or by ingestion of a BFDV-infected psittacine bird.
290
291 The Australian magpie (*Gymnorhina tibicen*) and Australian raven (*Corvus coronoides*) are both
292 members of the superfamily Corvoidea within the order Passeriformes. BFDV DNA was detected
293 in 33.3% of the 15 members of the superfamily Corvoidea tested in our study. Magpies mostly
294 forage on the ground and eat mainly annelids and small arthropods, while ravens are omnivorous,
295 eating plant matter, vertebrates, invertebrates and carrion [36]. Both species are known to prey on
296 nestlings [36]. Hence the most likely sources of BFDV for members of the Corvoidea would be
297 ingestion of BFDV-infected psittacine birds, ingestion of BFDV-contaminated insect vectors or
298 exposure to a BFDV-contaminated environment. Of the 24 birds belonging to the order
299 Passeriformes tested in our study, BFDV DNA was only detected in members of the superfamily
300 Corvoidea. While sample sizes for each family were very small, this result could suggest that other
301 passerine families may be resistant to BFDV infection. Furthermore, BFDV DNA was not detected
302 in the orders Columbiformes (pigeons and doves), Gruiformes (cranes and rails), Falconiformes
303 (falcons), Procellariiformes (pelagic seabirds) and Charadriiformes (shorebirds), suggesting either
304 lack of exposure of these species to BFDV, or potentially resistance of these orders to BFDV
305 infection. However sample sizes were again small, so the susceptibility of different non-psittacine
306 species to BFDV infection requires further investigation.
307

BFDV DNA was detected in 37.0% of the 192 wild birds and 50.0% of the 18 captive birds tested, providing evidence that BFDV is widespread and circulating at high prevalence amongst the avian fauna of Australia. Although the 210 liver samples tested in our study form a biased sample population, as all of these birds had either died or were euthanised due to the severity of their injuries or illness, the prevalence of BFDV infection was comparable to that seen in previous studies. We detected BFDV DNA in 30% of 20 Victorian Crimson rosellas, while another recent study detected BFDV DNA in 34.5% of 84 wild Crimson rosellas in south-eastern Australia [9]. Likewise Raidal *et al.* (1993) reported a seroprevalence of 41 - 94% within wild cockatoo flocks in New South Wales in the early 1990s using a haemagglutination inhibition assay [5], while we detected BFDV DNA in 67.7% of 31 Victorian cacatuids.

This investigation suggests that BFDV is more common in non-psittacine avian hosts than previously recognised, with BFDV DNA being detected in 20.0% of non-psittacine birds, even in species with no clear ecological association with psittacine species, and in all cases without any detectable clinical signs of PBFD. Further investigation is necessary to answer questions about viral shedding and the ability of non-psittacine host species to transmit BFDV to other birds and the environment, as well as the clinical significance of infection in non-psittacine hosts, and the ecological significance both for the non-psittacine bird populations involved and the potential threat to endangered psittacine species. This investigation has detected the presence of BFDV DNA in hepatic tissue from non-psittacine birds, but it is not known whether this BFDV DNA represents intact and replicating BFDV virions – hence further investigation, which incorporates haemagglutination assays as a better measure of viral viability, replicative competency and shedding, is needed. As the presence of haemagglutination inhibiting antibody titres is a strong negative predictive indicator for PBFD [4], serological studies should also form an integral component of future research to verify and expand on the results presented here, along with full genome sequencing to further investigate phylogenetic relationships. It would also be of value to

screen samples with the primer set targeting the BFDV replicase gene [37], as it is possible that the PCR primers differentially targeting the BFDV capsid and replicase genes have different sensitivies and result in differing prevalence estimates. This work would also enable easier comparison of BFDV haplotypes being sequenced by various research groups, as well as easier consolidation of prevalence estimates. The hypothesis of insects acting as vectors of infective BFDV virions and BFDV transmission via their ingestion also requires further investigation.

Eradication of BFDV is not possible, but management programs can assist in reducing the impact of the disease on threatened parrot populations [1]. The only disinfectants known to inactivate the non-enveloped and extremely resistant circoviruses are those containing the oxidising agent potassium peroxymonosulphate as their main ingredient, such as Virkon®S or ViralFX™ [15]. In light of the findings of the present investigation, which suggest that any bird could be a potential carrier of BFDV, it would be sensible for veterinary clinics, hospitals and wildlife rehabilitation facilities that provide care for birds that are susceptible to PBFD to consider the use of such disinfectants between examination of all birds, regardless of species and clinical presentation. In addition to these disinfection measures, separate examination and treatment facilities should be considered for psittacine species threatened by BFDV.

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Conflicts of interest: The authors declare that there is no conflict of interest.

Ethical statement

All samples were collected with approval from The University of Melbourne's Faculty of Veterinary and Agricultural Sciences Animal Ethics Committee, Ethics ID #1413397.

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462 **Table 1: Estimated prevalence of BFDV infection based on PCR detection of ORF C1 BFDV DNA from liver samples by species, family and**
463 **order.**
464

Bird species (common name)	Scientific name	Number of positive wild birds/total number of wild birds tested	Number of positive captive birds/total number of captive birds tested	Total BFDV PCR positives as a proportion of total number of birds tested (95% C. I.)	Number of positive birds/Total number of birds tested and estimated prevalence (%) by family (95% C. I.)	Number of positive birds/Total number of birds tested and estimated prevalence (%) by order (95% C. I.)
Australian king parrot	*Alisterus scapularis*	15/28	1/2	53.3% (35.9-70.2)	Psittacidae 38/74 51.4% (40.1-62.5)	Psittaciformes 59/105 56.2% (46.6-65.4)
Crimson rosella	*Platycercus elegans*	5/18	1/2	30% (13.6-51.7)		
Rainbow lorikeet	*Trichoglossus moluccanus*	3/5	0/0	60% (20.9-90.6)		
Eastern rosella	*Platycercus eximius*	6/11	0/0	54.5% (27.0-80.0)		
Orange-bellied parrot	*Neophema chrysogaster*	0/0	1/1	100% (14.7-100)		
Musk lorikeet	*Glossopsitta concinna*	2/2	0/0	100% (33.3-100)		
Scaly-breasted lorikeet	*Trichoglossus chlorolepidotus*	0/0	4/4	100% (55.5-100)		
Scarlet-chested parrot	*Neophema splendida*	0/0	0/1	0% (0-85.3)		
Sulphur-crested cockatoo	*Cacatua galerita*	12/17	0/0	70.6% (47.0-87.8)	Cacatuidae 21/31 67.7% (50.3-82.1)	
Galah	*Eolophus roseicapilla*	4/7	0/0	57.1% (23.5-86.1)		
Gang gang	*Callocephalon fimbriatum*	3/3	0/0	100% (46.4-100)		
Little corella	*Cacatua sanguinea*	1/1	0/0	100% (14.7-100)		
Long-billed corella	*Cacatua tenuirostris*	0/1	0/0	0% (0-85.3)		
Cockatiel	*Nymphicus hollandicus*	0/0	1/1	100% (14.7-100)		
Yellow-tailed black cockatoo	*Zanda funerea*	0/1	0/0	0% (0-85.3)		
Tawny frogmouth	*Podargus strigoides*	5/23	0/0	21.7% (8.8-41.3)	Podargidae 5/23 21.7% (8.8-41.3)	Caprimulgiformes 5/23 21.7% (8.8-41.3)
Laughing kookaburra	*Dacelo novaeguineae*	4/13	0/0	30.8% (11.4-57.7)	Alcedinidae 5/14 35.7% (15.2-61.6)	Coraciiformes 5/14 35.7% (15.2-61.6)
Sacred kingfisher	*Todiramphus sanctus*	1/1	0/0	100% (14.7-100)		

Australian wood duck	*Chenonetta jubata*	0/4	0/0	0% (0-44.5)	Anatidae 1/14 7.1% (0.8-28.8)	Anseriformes 1/14 7.1% (0.8-28.8)
Pacific black duck	*Anas superciliosa*	0/4	0/0	0% (0-44.5)		
Chestnut teal	*Anas castanea*	0/1	0/3	0% (0-44.5)		
Australian shelduck	*Tadorna tadornoides*	0/1	0/0	0% (0-85.3)		
Hardhead duck	*Aythya australis*	0/0	1/1	100% (14.7-100)		
Southern boobook	*Ninox boobook*	1/6	0/0	16.7% (1.9-55.8)	Strigidae 2/7 28.6% (6.5-64.8)	Strigiformes 3/8 37.5% (11.9-70.5)
Powerful owl	*Ninox strenua*	1/1	0/0	100% (14.7-100)		
Barn owl	*Tyto alba*	1/1	0/0	100% (14.7-100)	Tytonidae 1/1 100% (14.7-100)	
Common bronzewing	*Phaps chalcoptera*	0/2	0/0	0% (0-66.7)	Columbidae 0/6 0% (0-33.0)	Columbiformes 0/6 0% (0-33.0)
Crested pigeon	*Ocyphaps lophotes*	0/2	0/0	0% (0-66.7)		
Peaceful dove	*Geopelia placida*	0/0	0/1	0% (0-85.3)		
Spotted turtle dove (Introduced)	*Spilopelia chinensis*	0/1	0/0	0% (0-85.3)		
Australian white ibis	*Threskiornis moluccus*	1/5	0/0	20% (2.3-62.9)	Threskiornithidae 1/5 20% (2.3-62.9)	Pelecaniformes 1/6 16.7% (1.9-55.8)
White-faced heron	*Egretta novaehollandiae*	0/0	0/1	0% (0-85.3)	Ardeidae 0/1 0% (0-85.3)	
Purple swamphen	*Porphyrio porphyrio*	0/5	0/0	0% (0-37.9)	Rallidae 0/5 0% (0-37.9)	Gruiformes 0/5 0% (0-37.9)
Wedge-tailed eagle	*Aquila audax*	0/1	0/0	0% (0-85.3)	Accipitridae 1/2 50% (6.1-93.9)	Accipitriformes 1/2 50% (6.1-93.9)
Brown goshawk	*Accipiter fasciatus*	1/1	0/0	100% (14.7-100)		
Peregrine falcon	*Falco peregrinus*	0/1	0/0	0% (0-85.3)	Falconidae 0/1 0% (0-85.3)	Falconiformes 0/1 0% (0-85.3)
Short-tailed shearwater	*Ardenna tenuirostris*	0/1	0/0	0% (0-85.3)	Procellariidae 0/1 0% (0-85.3)	Procellariiformes 0/1 0% (0-85.3)

Australian magpie	*Gymnorhina tibicen*	4/11	0/0	36.4% (13.7-65.2)	Cracticidae 4/12 33.3% (12.5-61.2)	Passeriformes 5/24 20.8% (8.4-39.8)
Pied currawong	*Strepera graculina*	0/1	0/0	0% (0-85.3)		
Australian raven	*Corvus coronoides*	1/3	0/0	33.3% (3.9-82.3)	Corvidae 1/3 33.3% (3.9-82.3)	
Superb lyrebird	*Menura novaehollandiae*	0/1	0/0	0% (0-85.3)	Menuridae 0/1 0% (0-85.3)	
Yellow-tufted honeyeater	*Lichenostomus melanops*	0/0	0/1	0% (0-85.3)	Meliphagidae 0/1 0% (0-85.3)	
Satin bowerbird	*Ptilonorhynchus violaceus*	0/1	0/0	0% (0-85.3)	Ptilonorhynchidae 0/1 0% (0-85.3)	
Red-browed finch	*Neochmia temporalis*	0/1	0/0	0% (0-85.3)	Estrildidae 0/1 0% (0-85.3)	
Grey shrike-thrush	*Colluricincla harmonica*	0/1	0/0	0% (0-85.3)	Colluricinclidae 0/1 0% (0-85.3)	
Striated pardalote	*Pardalotus striatus*	0/1	0/0	0% (0-85.3)	Pardalotidae 0/1 0% (0-85.3)	
Brown thornbill	*Acanthiza pusilla*	0/1	0/0	0% (0-85.3)	Acanthizidae 0/1 0% (0-85.3)	
Bassian thrush	*Zoothera lunulata*	0/1	0/0	0% (0-85.3)	Turdidae 0/2 0% (0-66.7)	
Eurasian blackbird (Introduced)	*Turdus merula*	0/1	0/0	0% (0-85.3)		
Hooded plover	*Thinornis cucullatus*	0/1	0/0	0% (0-85.3)	Charadriidae 0/1 0% (0-85.3)	Charadriiformes 0/1 0% (0-85.3)
TOTALS	**BFDV DNA detected from 23 of the 50 bird species tested**	**71/192 37.0% (30.4-44.0)**	**9/18 50.0% (28.4-71.6)**	**80/210 38.1% (31.7-44.8)**	**BFDV DNA detected from 11 of the 25 families tested**	**BFDV DNA detected from 8 of the 13 orders tested**

Wild birds were presented to the AWHC for veterinary care, while captive birds were part of the Healesville Sanctuary collection.

Figure 1: Maximum likelihood phylogenetic tree of partial circovirus ORF C1 nucleotide sequences, constructed using PhyML from an alignment of 31 circovirus sequences detected in this study (highlighted in bold) and 59 avian circovirus sequences retrieved from GenBank (with labels at branch tips indicating the host bird species and GenBank accession numbers), using Geneious version 9.0 (Biomatters) [20]. Branching with greater than 50% support from 1000 bootstrap replicates is indicated at node points. Horizontal distances correspond to genetic distances; vertical distances are arbitrary. See Table S1 for details on the host bird sources for the sequences obtained in this study.

Figure

Click here to download Figure Fig 1 Circovirus tree 31st Dec 2016.pdf

Table S1: BLAST* analysis of partial BFDV capsid protein gene sequences obtained from DNA extracted from liver samples

Source of sample	Origin	Aetiology of presentation	Clinical PBFD signs	GenBank accession number	Fragment size (bp)	Highest similarity to (GenBank accession number)	Total BLAST score	Query coverage (%)	Maximum nucleotide identity (%)
Australian king parrot 150371	Healesville	HBC†, fractured radius + ulna; euthanised on admission	None noted	KY410348	447	BFDV wild Crimson rosella 18-NSW-2006 (KJ953878)	715	100	96
Australian king parrot B30773	HS‡ Collection, originally from private breeder	Emaciated, plasmacytic enteritis, chronic biliary hyperplasia	Scruffy plumage	KY410349	402	BFDV wild Crimson rosella hybrid Moyhu-VIC-2011 (KJ953849)	697	100	98
Australian magpie 150034	Healesville	Neurological signs, emaciated, lymphoplasmacytic enteritis with crypt necrosis; in captivity for 2 days prior to euthanasia	None noted	KY410350	422	BFDV Musk lorikeet 150065 VIC 2015 (KX449322)	752	100	99
Australian magpie 150411	Healesville	HBC, pulmonary haemorrhage, R§ leg paresis; euthanised on admission	None noted	KY410351	410	BFDV AUS Galah 2004 Perth, WA (KF385431)	719	100	98
Australian magpie 150490	Healesville	Unknown, internal haemorrhage + perforated gizzard; died overnight the day after rescue	None noted	KY410352	383	BFDV AUS Galah 2004 Perth, WA (KF385431)	608	100	95
Australian magpie 150679	Wesburn	HBC, radial + ulnar fractures, pulmonary contusions; euthanised the day after admission	None noted	KY410353	415	BFDV Musk lorikeet 150065 VIC 2015 (KX449322)	728	100	98
Australian raven 150549	Woori Yallock	Emaciated, neurological signs, neoplasia (disseminated malignant round cell tumour, possibly lymphoblastic lymphoma); euthanised on admission	None noted	KY410354	398	BFDV AUS Galah 2004 Perth, WA (KF385431)	614	100	94
Australian white ibis 150542	Badger Creek	Attacked by falcon, severe internal haemorrhage; dead on arrival	None noted	KY410355	444	BFDV AUS Galah 2004 Perth, WA (KF385431)	664	99	94
Brown goshawk 150703	Badger Creek	Humeral fracture of unknown cause, internal haemorrhage, emaciated; euthanised on admission	None noted	KY410356	392	BFDV wild Crimson rosella hybrid Moyhu-VIC-2011 (KJ953849)	702	100	99

Cockatiel B40091	HS Collection	Avian chlamydiosis, poor body condition	None noted	KY410357	399	BFDV AUS Galah 2004 Perth, WA (KF385431)	610	100	94
Crimson rosella 150638	Marysville	Dog attack, open ulnar fractures; euthanised on admission	None noted	KY410358	420	BFDV wild Crimson rosella hybrid Moyhu-VIC-2011 (KJ953849)	754	100	99
Crimson rosella 150890	Mount Toolebewong	Avian chlamydiosis, emaciated with diarrhoea; euthanised on admission	Scruffy feathers with feather loss round ventral neck	KY410359	399	BFDV wild Crimson rosella hybrid Moyhu-VIC-2011 (KJ953849)	693	100	98
Crimson rosella B50208	HS Collection, originally from private breeder	Undetermined	Flaky beak, scruffy plumage	KY410360	415	BFDV wild Crimson rosella hybrid Moyhu-VIC-2011 (KJ953849)	756	100	99
Eastern rosella 150115	Mitcham	Unable to fly, suspected PBFD; euthanised on admission	Missing primary flight and tail feathers, many abnormal feathers – fragile, easily epilated, pinched/bent shafts	KY410361	447	BFDV wild Crimson rosella hybrid Moyhu-VIC-2011 (KJ953849)	771	100	98
Galah 150503	Kilsyth	HBC, fractured femur, ruptured liver, emaciated; euthanised on admission	None noted	KY410362	447	BFDV AUS Galah 2004 Brisbane, QLD (KF385435)	815	100	99
Gang gang 150446	Chirnside Park	HBC, head trauma, internal haemorrhage, chronic shoulder subluxation; euthanised on admission	None noted	KY410363	407	BFDV wild Crimson rosella 18-NSW-2006 (KJ953878)	658	100	96
Laughing kookaburra 150445	Kinglake Central	Unable to fly, emaciated, weak, anorexic, dehydrated, anaemic, hypoproteinaemic, septicaemic with hepatic necrosis, heterophilic ventriculitis with ulceration, intralesional bacteria and nematode parasites, and heterophilic enteritis with crypt abscesses; in captivity for 2 days prior to euthanasia	None noted	KY410364	447	BFDV AUS Galah 2004 Brisbane, QLD (KF385435)	815	100	99
Laughing kookaburra 150447	Healesville	HBC, fractured orbit, corneal injury, intracoelomic haemorrhage; euthanised on admission	Normal plumage but scaly crusting of skin over ventrum	KY410365	447	BFDV wild Crimson rosella 18-NSW-2006 (KJ953878)	704	100	95

Little corella 150485	Woori Yallock	Flew into window, skull fracture, chest trauma, severe ataxia; euthanised on admission	Dirty plumage, clubbed feathers over flanks	KY410366	447	BFDV AUS wild Long-billed corella Melb, VIC 2010 (KF385423)	787	100	98
Musk lorikeet 150065	Ringwood	Unknown, muscle atrophy round R pectoral girdle; euthanised the day after rescue	None noted	KY410367	447	BFDV Musk lorikeet 150065 VIC 2015 (KX449322)	826	100	100
Orange-bellied parrot B01871	HS Collection	Dyspnoea, bronchopneumonia with mycotic necrogranuloma, cutaneous mite infestation, renal tubular inclusions consistent for avian polyomavirus infection	Scruffy plumage	KY410368	444	BFDV AUS Galah 2004 Perth, WA (KF385431)	667	99	94
Powerful owl 150420	St Kilda	Columbid herpesvirus 1, small intestinal enteritis with mucosal necrosis, pulmonary haemorrhage; died overnight after admission	None noted	KY410369	444	BFDV wild Sulphur-crested cockatoo Darwin, NT (AF311301)	806	100	99
Rainbow lorikeet 141266	Badger Creek	HBC, head trauma, liver rupture; euthanised on admission	None noted	KY410370	447	BFDV Musk lorikeet 150065 VIC 2015 (KX449322)	793	100	99
Scaly-breasted lorikeet B10154	HS Collection	Glomerulonephritis, renal gout, metastatic proventricular mineralisation, chronic hepatitis with biliary stasis	None noted	KY410371	416	BFDV AUS Galah 2004 Perth, WA (KF385431)	702	100	97
Scaley-breasted lorikeet B50250	HS Collection	Avian chlamydiosis, intraepithelial basophilic inclusion bodies in the small intestine with mucosal epithelial cell necrosis and crypt hyperplasia	None noted	KY410372	447	BFDV Musk lorikeet 150065 VIC 2015 (KX449322)	793	100	99
Scaly-breasted lorikeet B50355	HS Collection, recent fledgling	Avian chlamydiosis, poor body condition, depressed, proliferative enteropathy, ventriculitis with mucosal necrosis	None noted	KY410373	412	BFDV AUS Galah 2004 Perth, WA (KF385431)	723	100	98

Scaly-breasted lorikeet B50365	HS Collection, recent fledgling	Unable to fly, poor body condition, perihepatic haematoma	Missing primary flight and tail feathers, stunted new feathers, pinched quills, yellow discolouration of feathering. Intracytoplasmic basophilic inclusions characteristic of circovirus infection in lymphoid cells in bursa of Fabricius and spleen, epithelial cell hyperplasia and dysplasia with intranuclear circoviral inclusions in feather follicles.	KY410374	447	BFDV Musk lorikeet 150065 VIC 2015 (KX449322)	787	100	98
Southern boobook owl 150510	Jamieson	Dog attack; euthanised on admission	None noted	KY410375	408	BFDV AUS Galah 2004 Brisbane, QLD (KF385435)	743	100	99
Sulphur-crested cockatoo 150738	Lilydale	Clinical PBFD, emaciated, weak, unable to fly, haemorrhagic enteritis with piriform protozoan infestation, overgrowth of *Macrorhabdus ornithogaster* in ventriculus, ectoparasite infestation; euthanised on admission	Dirty plumage, lack of powder down, pinched feather quills, overgrown upper beak	KY410376	420	BFDV AUS Galah 2004 Perth, WA (KF385431)	737	100	98
Tawny frogmouth 150448	Surrey Hills	Unknown; in captivity for a day prior to death	None noted	KY410377	444	BFDV AUS Galah 2004 Perth, WA (KF385431)	667	99	94
Tawny frogmouth 150712	Eildon	Emaciated, anaemic, hypoproteinaemic, mild nematodiasis, undetermined; in captivity for a day prior to death	None noted	KY410378	409	BFDV AUS Galah 2004 Perth, WA (KF385431)	745	100	99

[*] BLAST = Basic Local Alignment Search Tool
[†] HBC = hit by car
[‡] HS = Healesville Sanctuary
[§] R = right