Cockatiels (*Nymphicus hollandicus*) have their own Beak and Feather Disease Virus (BFDV)

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Introduction

Psittacine Beak and Feather Disease (PBFD) is known to occur in a wide range of psittacine species, however there are no scientific or credible anecdotal reports of PBFD occurring in the cockatiel (*Nymphicus hollandicus*) despite it being one of the world's most commonly kept companion bird species. Consequently this has resulted in speculation that the species may have some innate resistance to beak and feather disease virus (BFDV) infection. However, we provide histological, DNA sequencing and serotyping evidence of BFDV infection in cockatiels, thus confirming that the species is susceptible to BFDV infection. Maximum parsimony and Bayesian analysis of two cockatiel isolates placed them into a clade genetically distinct from other BFDV sequences and HI cross-reactivity analysis also demonstrated evidence of antigenic variation in one of the cockatiel BFDV isolates when it was used as the antigen against known positive BFDV antisera. A survey of cockatiels (n=88) at commercial aviaries failed to detect serological or PCR evidence of BFDV infection but serological cross-reactivity results and phylogenetic analysis of the nucleotide sequences indicated that the cockatiel virus isolates may be serologically and genetically different to other BFDV isolates.

Materials and Methods

In order to investigate the apparently low rate of BFDV infection in cockatiels we decided to survey cockatiels at 3 commercial aviaries in Perth, Western Australia using PCR, as well as haemagglutination (HA) and haemagglutination-inhibition (HI) assay. In addition, BFDV DNA samples from the feathers of 2 cockatiels submitted to us for BFDV diagnostic testing were amplified by PCR, sequenced and analysed.

Feathers from two cockatiels (isolates 05-106 and 05-726) which had lesions consistent suggestive of PBFD were submitted to our laboratory. The tail and primary flight feathers of cockatiel 05-106 were absent and there were areas of patchy feather loss distributed randomly through the powder-down feathers and the feathers of the body. The feathers submitted tested BFDV-positive by PCR and HA were used for subsequent PCR analysis and DNA sequencing as described below. A formalin-fixed

feather follicle skin biopsy, submitted along with the feather of 05-106, was also processed by routine histology methods, stained with haematoxylin and eosin and examined by light microscopy for the presence of characteristic inclusion bodies and BFDV infection was confirmed by immunohistochemistry.

A survey of cockatiels in commercial aviaries was also conducted. Blood and feathers from 88 cockatiels at three commercial aviaries that had a laboratory confirmed history of housing PBFD-affected psittacine birds were taken for testing. Blood was collected by venepuncture of the jugular or cutaneous ulnar vein and spotted onto filter paper (Whatmann, No. 3), then allowed to air dry at room temperature as described by Riddoch *et al*. (1996). Feathers were plucked and placed into clean 1.5 mL microcentrifuge tubes or zip-lock bags. PCR and HI was performed on blood and feathers were used for HA testing.

Immunohistochemical (IHC) Staining.

IHC staining using primary monoclonal antibodies to recombinant BFDV capsid protein (Stewart et al 2007) and a horseradish peroxidase (HRP) conjugated secondary antibody were performed on tissue sections from case 05-106. Briefly, 5 μm sections of formalin-fixed and paraffin embedded feather tissue were cut using a Leica RM 2135 microtome, placed onto glass slides, de-waxed 3 times in xylene for 3 min and re-hydrated using decreasing ethanol concentrations and a final wash in Tris buffer for 3 min. Endogenous peroxides were quenched using 0.3% (v/v) H_2O_2 in methanol for 5 min and then washed in Tris buffer. Slides were incubated with a 1:500 dilution of the primary monoclonal antibody in Tris buffer for 10 minutes at room temperature and unbound antibody was then removed by triplicate washes each for 3 min in Tris buffer before incubating with HRP conjugated EnVision anti-mouse (Dako) at room temperature for 30 min. The slide was washed as before and then antigen-antibody complexes visualised with the chromagen diaminobenzidine (DAKO® DAB chromagen). Imaging was performed using an Olympus BX 13 microscope and digital camera accessory.

Polymerase Chain Reaction (PCR)

Viral DNA was extracted from feathers and PCR was carried out using methods similar to those described by Ypelaar *et al*. (1999). Reactions were carried out in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf). PCR products were visualised on a 1% agarose gel with the addition of 0.001% ethidium bromide, run at 90V for 30 minutes.

Haemagglutination (HA) and Haemagglutination Inhibition (HI) Assays

HA and HI assays were carried out as described by Raidal *et al.*, (1993b). Antigen purified from the feathers of a cockatoo with PBFD was used in the HI assay. Plasma, serum or dried blood spots on filter paper were used for testing from the birds.

Cross-reactivity Assays

An additional set of HI assays was performed to investigate the possible existence of a cockatiel-specific BFDV serotype. HI assays were performed as usual, except that the antigen and negative control was virus eluted from the feather of a cockatiel that had tested positive for BFDV by PCR and HA. Sera from seven different psittacine bird species including two short-billed corellas (*Cacatua sanguinea*), a sulphur-crested cockatoo (*Cacatua galerita*), two rainbow lorikeets (*Trichoglossus haematodus*), one corella (*Cacatua tenuirostris*), one red lory (*Eos bornea*) and one galah–corella hybrid with known HI titres were reacted against virus eluted from cockatiels 05-106 and 05-726. Sera

from all cockatiels sampled at the commercial aviaries was also tested against BFDV eluted from the feather of cockatiel 05-106.

Nucleotide Sequence Determination and Analysis

PCR products were purified using an Axyprep PCR cleanup kit (Axygen), according to the manufacturer's instructions. Dideoxynucleotide sequencing was carried out using an ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, except that the reaction volume was reduced to 10 µL and the annealing temperature used when sequencing the 1478 bp products was reduced to 50ºC. Sequencing reactions consisted of 2 µL of reaction buffer (containing Tris-HCl, $MgCl_2$, fluorescently-labelled dNTPs and AmpliTaq DNA polymerase, concentrations not supplied; Perkin Elmer), 1 µL of 5x sequencing buffer (composition not supplied, Perkin Elmer), 10-20 ng of 717 bp PCR product or 20-40 ng of the 1479 bp PCR product, then made up to a total of 10 µL with ultrapure water (Fischer Biotec).

DNA sequences were determined using an Applied Biosystems 3730 DNA Analyser and edited using Sequence Scanner v1.0 (Applied Biosystems) and GeneTool Lite (BTI Software). Edited sequences were analysed using MEGA 3.1 (Kumar *et al.*, 2004). Neighbour-joining (NJ), maximum parsimony (MP) and Bayesian trees were constructed with 1000 bootstrap cycles for NJ and MP trees.

Results

Survey of Cockatiels for Evidence of BFDV and Cross-reactivity Assays

Of the 88 cockatiels tested, none were positive for BFDV by PCR or HA and none had detectable antibodies to BFDV. None of the cockatiels surveyed had detectable HI activity against BFDV eluted from a cockatiel feather. Six of the 8 known anti-BFDV HI positive sera tested inhibited HA eluted from the feather of cockatiel 05-106 and similarly 5 of the 8 sera inhibited HA eluted from isolate 05-726. Polyclonal chicken anti-BFDV antibody did not inhibit agglutination by either cockatiel virus isolate (Table 1).

Sequencing and Analysis of PCR-positive Samples from Cockatiels

Primer sets P2/P4 and SeqP5/SeqP10 amplified overlapping 717 bp and 1497 bp fragments of the BFDV genome, respectively, from samples 05-106 and 05-726. Analysis of the sequences revealed that both sequences were 1993 nt long and had identical features to other described circoviruses. A potential stem-loop structure, formed between bases 1976-1993 and 1-12, as well as a repeated octanucleotide motif (GGGCACCG) were present immediately downstream of the stem-loop. Potential polyadenation signals were present in both sequences at identical positions, CATAAA between nt 1019-1024 on the viral strand and AATAAA on the complementary strand between nt 758-763 (nt 1231-1236 of the viral strand). A TATA box was also present, between nt 86-89 of the viral strand. The area of the complementary strand between nt 60-207, containing putative nuclear localisation signals as described by Heath *et al.* (2006) was highly conserved across all sequences analysed, including the 2 cockatiel sequences.

Comparison of Full-length Sequences

Distances between cockatiel sequences and other BFDV sequences varied between 3.2-15.5% (05-106) and 3.8-14.5% (05-726) at the nucleotide level and between 3.8-19% (05-106) and 5.2-19.2% (05-726) at the amino acid level. Neighbour joining phylogenetic analysis showed that both cockatiel sequences (isolate 05-106 and 05-726 GenBank Accession Nos. EF457974 and EF457975, respectively) clustered

within cockatoo and galah isolates, however Maximum parsimony (not shown) and Bayesian analysis grouped the cockatiel isolates distinctly separately (Figure 1). Bayesian analysis also identified 3 distinct clades within the sequences: the cockatiels comprised clade number 1; the cockatoos and galahs plus 3 African grey parrots (AY521236, AY450443 and AY450435), a white-bellied caique (AY450434) and a Cape parrot (AY450439) made up clade 2 and the remaining clade was made up of all *Agapornis* sp. isolates, the remaining African grey parrot and *Poicephalus sp.* plus a single rainbow lorikeet and a single Indian ringneck isolate.

A plot of CpG islands within the cockatiel sequences showed high proportions of CpG motifs throughout most of the sequence. Gardiner-Garden and Frommer, (1987), defined a candidate CpG island as having a Y-value of >0.6 on the CpG plot and a GC content of >50%. As such, the first 200 bases of the sequence, then bases 240-650, 680-1160, 1210-1380 and 1760-1993 contain candidate CpG islands. Particular points of interest were spikes in the CpG plot at nt 280-400, 672, 1167 and 1660-1880 of the viral strand. The spikes at nt 672, 1167 and 1660-1760 are less likely to be significant, though, as the GC content in these regions is less than 50%. The complementary strand had candidate CpG islands within the first 30 nt and between nt 420-600, 640-1120, 1150-1570 and 1575-1993. There were large spikes in the CpG plot of the complementary strand at around nt 630 and nt 1130, but the GC content was below 50% at these points and hence they are not considered candidate CpG islands. The whole sequence was GC-rich, as the GC content was only less than 50% between bases 200-240, 650-680, 1160-1210 and 1380-1760 of the viral strand. Comparison of CpG plots derived from the cockatiel isolates demonstrated subtle differences compared to plots derived from published BFDV sequences from a sulphur-crested cockatoo (AF080560), rainbow lorikeet (AF311299) and peach-faced lovebird (AF311296). GC density plots for these isolates demonstrated minimal variation between the 4 isolates.

ORFV1

Sequences of both cockatiel isolates had a start codon of ATG located at position 131. The stop codon for both isolates was TGA, located at nt 997. The predicted size of ORFV1 was 867 nt. Distances between cockatiel sequences and other BFDV sequences varied between 2.5-11.9% (05-106) and 2.4-11.1% (05-726) at the nucleotide level and between 2.2-11% (05-106) and 1.1-10.4% (05-726) at the amino acid level. Phylogenetic analysis showed that both cockatiel sequences clustered closest to, but separate from, cockatoo and galah isolates (Figure 1).

ORFC1

Sequences of both cockatiel isolates had a putative start codon at nt 16 (CTG) of the complementary strand (or nt 1978 of the viral strand), as per Bassami *et al.* (2001). The stop codon for both isolates was a TAA at nt 757 of the complementary strand (nt 1235 of the viral strand Distances between cockatiel sequences and other BFDV sequences varied between 2.0-18.9% (05-106) and 5.9-19.1% (05-726) at the nucleotide level and between 6.0-28.5% (05-106) and 6.8-27.4% (05-726) at the amino acid level. Phylogenetic analysis showed that both cockatiel sequences clustered within a clade of cockatoo and galah isolates. Alignment of translated amino acid sequences showed that 121 of 260 amino acids were conserved across all the isolates examined and 133 of 260 were variable. The areas between aa68-83, 94-97 and 228-241 were especially variable but the significance of this is unknown.

Discussion

PBFD has been confirmed in more than 60 psittacine species and it is highly likely that all are susceptible (Pass and Perry, 1985, Rahaus and Wolff, 2003, Albertyn *et al.*, 2004, Ritchie *et al.*, 1989). Surveys have been carried out in both wild and captive psittacine populations and reported virus

prevalence rates vary between 10-94%, depending on the method of detection (McOrist *et al.*, 1984, Raidal *et al.*, 1993a, Rahaus and Wolff, 2003, Khalesi *et al.*, 2005).

Given the wide range of *Psittaciforme* species reported to be susceptible, it is curious that the cockatiel (*Nymphicus hollandicus*) is greatly underrepresented in both the scientific and lay literature. Indeed we know of no published reports of PBFD in cockatiels even though the species is one of the most commonly kept companion bird species worldwide. It seems the only evidence of BFDV infection occurring in the cockatiel was a diagnosis made by polymerase chain reaction (PCR) in our own laboratory (Khalesi *et al.,* 2005).

The data provides histological, DNA sequence and serotyping evidence of BFDV infection in cockatiels, thus confirming that the species is susceptible to BFDV infection. Immunohistochemical staining and DNA sequence data and CpG analysis demonstrated antigenic and genetic relationship with BFDV isolates obtained from other *Psittaciforme* species. However, maximum parsimony and Bayesian analysis of the cockatiel isolates placed them into a clade genetically distinct from other BFDV sequences and HI cross-reactivity analysis also demonstrated evidence of antigenic variation in one of the cockatiel BFDV isolates when it was used as the antigen against known positive BFDV antisera.

BFDV is a genetically diverse virus and there have been numerous phylogenetic studies on the now many isolates that have had their complete nucleotide sequences determined. Broad genotype lineages aligned to the major Families of psittacine birds namely the cockatoos, loriids and parrots have been demonstrated but the biological significance of this clustering has not been well understood. There have been few papers that have investigated antigenic variation in the virus but BFDV isolates harvested from a diverse range of psittacine genera were found to be antigenically similar by Ritchie *et al.* (1990) and antigen derived from the feathers of diseased cockatoos has, until now, proven to be useful for detecting antibody to BFDV using HI assay. Within the *Cacatuidae* there are 6 genera and 21 species and within the *Psittacidae* there are 78 genera and 332 species. Numerous papers have found HI assay suitable for detecting anti-BFDV antibodies in sera from a large proportion of these 353 species (Raidal *et al.,* 1993a,b; Raidal and Cross 1994a; Ritchie *et al.,* 1991; Riddoch *et al.,* 1996; Khalesi *et al.,* 2005). Khalesi *et al.* (2005) demonstrated no evidence of any antigenic serotypes by HI cross-reactivity studies using feather and blood samples obtained from a range of psittacine bird species and an identical technique to that described in this present paper. The fact that HI antibodies against a single antigen has been successfully used to detect anti-BFDV in a range of psittacine bird species is good evidence that there is a considerable degree of cross reactivity between the different genotypes that infect cockatoos, lorikeets and parrots.

The low reported incidence of BFDV infection in cockatiels and our negative serological survey results is somewhat puzzling for such a supposedly common and infectious virus that all *Psittaciformes* are presumed to be susceptible to. It could be that all of the cockatiels we surveyed in this present paper were naïve to infection, and truly antibody negative. However, they were from commercial aviaries that had a high turn-over of a wide variety of psittacine bird species many of which we knew from clinical observations were expressing clinical signs of PBFD. Typically the birds in such establishments have a high incidence of BFDV infection, PBFD and a high HI antibody prevalence with budgerigars (*Melopsittacus undulatus*) and lovebirds (*Agapornis* spp.) having the highest rates of infection (Khalesi *et al.,* 2005).

According to published epidemiologic data an expected seroprevalence of 30-40% would be a conservative estimate (Raidal *et al.,* 1993a, Raidal and Cross 1994b; Khalesi *et al.,* 2005) of the expected seroprevalence within the population of cockatiels that we sampled and a sample size of 88 should have provided a 95% level of confidence of the estimate of the prevalence of infection

(Thrushfield 1986). Failure to detect any evidence of antibody in such a sample size provides strong evidence (95% confidence level) that the seroprevalence in the population of cockatiels we sampled was less than 5% (Thrushfield 1986). This is a very low figure in comparison with other *Psittaciforme* species. The lack of documented cases of cockatiels with PBFD in the literature along with the negative PCR and serological results obtained in our survey suggest that cockatiels are somewhat innately resistant to infection with common (eg cockatoo and lorikeet) BFDV isolates. However, the PCR results in individual cockatiels reported here and by Khalesi *et al.* (2005) along with the histological evidence of BFDV infection indicates that the species is susceptible to the BFDV isolate found in these cockatiels. Furthermore, the PCR results, DNA sequence analysis and HI cross-reactivity data provides evidence of a cockatiel-adapted BFDV which may be sufficiently different, genetically and antigenically, to most other BFDV isolates to be considered a separate strain of the virus. This is not surprising given evidence that avian circoviruses have coevolved with their host species (Ritchie *et al.,* 2003; Stewart *et al.,* 2006). *Psittaciforme* mitochondrial DNA phylogeny has placed the monotypic *Nymphicus* more closely related to the black cockatoos (*Calyptorynchus* and *Callocephalon*) and not the more distantly related white cockatoos (*Cacatua* and *Eolophus*) as was once thought (Brown and Toft, 1999) and the results presented in this present paper provide further support to this hypothesis.

There are some aspects of BFDV evolution and epidemiology that are difficult to explain. Based on the phylogenetic analysis (Figure 1), the cockatiel BFDV sequences are genetically distinct from those obtained from other psittacine species. However, the same method of analysis and other similar studies have also found similar distinct genetic differences between BFDV isolates from lovebirds, lorikeets and cockatoos, and cross-reactivity work has shown these not to be serologically distinct (Khalesi *et al*., 2005) and therefore it is difficult to clearly associate a specific mutation or genetic variation in the cockatiel sequences with any biological or antigenic characteristics.

Phylogenetic analysis of full length sequences and V1 sequences grouped the cockatiels separately to other isolates, but analysis of C1 sequences alone grouped the cockatiel sequences appropriately within a cockatoo clade (data not shown). Most studies thus far have focused on the C1 gene as the main determinant of the pathogenicity of the virus (Raue *et al.*, 2004; Heath *et al.*, 2004) but the fact that the C1 gene grouped within a known clade while the V1 gene and full sequences grouped separately suggests three things: firstly, that the V1 gene may have other functions than first thought; second, that parts of the sequence other than the C1 and V1 gene play a part in pathogenesis and third that host factors (such as the presence or absence of cell surface receptors for virus attachment or MHC presentation) play a significant role.

The second of these possibilities is supported by work with porcine circovirus 2 (PCV2) showing that CpG motifs play a role in the modulation of α-interferon expression (Hasslung *et al.,* 2003) and as such CpG motifs may play a similar role in modulating cytokines during the course of BFDV infection. Fenaux *et al.* (2003; 2004) demonstrated that when the capsid-coding region of PCV2 was cloned into the genomic backbone of PCV1, the resultant chimeric virus was less pathogenic than wild-type PCV2. This is not to say that the C1 gene and capsid protein are not significant in the pathogenesis of the disease. Mahe *et al.*, (2000) identified capsid epitopes unique to PCV1 and PCV2 and the presence of unique epitopes may occur with BFDV as well and may explain the variable cross-reactivity of the cockatiel isolates. Considering that there are currently no cell-culture techniques or any *in vitro* methods to propagate BFDV, the identification of these unique epitopes and other motifs suspected to play a role in pathogenesis will need to be carried out using such techniques as epitope mapping and infectivity studies using infectious clones. Likewise, infectivity studies with various mutant BFDV infectious clones and a wide range of psittacine species may be the only way to investigate the extent to which the genotype of an isolate affects its pathogenicity. More specifically, it may be the only way to investigate the relative susceptibility or resistance of cockatiels to other BFDV isolates and whether the cockatiel BFDV isolate is infectious to other psittacine species.

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Table 1. Haemagglutination inhibition cross-reactivity of known positive anti-BFDV (cockatoo) psittacine sera against virus eluted from the feathers of two cockatiels with PBFD

Antibody Source `	**Cockatoo BFDV**	**Cockatiel 05-106**	**Cockatiel 05-726**
Chicken anti-BFDV	+	Neg	Neg
Red Lory	+	Neg	Neg
Corella	+	+	Neg
Galah/Corella hybrid	+	+	+
Rainbow lorikeet	+	+	+
Rainbow lorikeet	+	+	+
Short billed corella	+	Neg	Neg
Short billed corella	+	+	+
Sulphur crested cockatoo	+	+	+

Figure 1. Bayesian phylogram constructed using full-length BFDV sequences demonstrating the distance between cockatiel (*Nymphicus hollandicus*) BFDV sequences (EF457974 and EF457975) in relationship to published BFDV sequences from other *Psittacidae* and *Cacatuidae*.