Sensitivity and specificity of HA, HI and PCR for detecting psittacine beak and feather disease virus (BFDV) testing.

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INTRODUCTION

Psittacine beak and feather disease (PBFD) continues to be a major cause of ill health in companion and wild birds throughout Australia and is listed as a key threatening process for several endangered psittacine bird species. The virus that causes the disease (BFDV) is a haemagglutinating circovirus which has permitted the development of haemagglutination (HA) and haemagglutination inhibition (HI) assays for the virus and antibody responses to infection, respectively (Raidal and Cross 1994a). These assays, as well as PCR testing based on the relatively conserved ORF-V1 (Ypelaar et al., 1999; Ritchie et al., 2003), are in wide use throughout Australia and the world for diagnosing infection (Raidal et al., 1993a; Sanada and Sanada, 2000). We decided to determine the limit of detection of our HA and PCR assays and compared the three tests on feather and blood samples sent to us for routine diagnostic testing.

MATERIALS AND METHODS

Samples

Feather and blood samples were submitted by referring veterinarians throughout Australia from 623 psittacine birds tentatively diagnosed with PBFD, or with a history of being in contact with PBFD-affected birds. Some samples were submitted to confirm absence of disease in quarantined birds. Blood samples were either collected onto filter paper as described by Riddoch et al., (1996) when long distance transportation was required, or collected into heparinised collection tubes for processing in our laboratory (Raidal et al., 1993a). Developing feathers or dried feathers with lesions were collected and transported unpreserved.

To compare feather and blood PCR testing a flock of 56 peach faced lovebirds recently imported into a pet shop was sampled by feather HA, feather PCR, blood PCR and HI antibody testing as described below.

Haemagglutination (HA) and haemagglutination inhibition (HI) assays

HA assays were performed on feather samples using galah (*Eolophus roseicapillus*) erythrocytes and BFDV antigen derived from a sulphur crested cockatoo (*Cacatua galerita*) as described by Raidal et al., (1993a). HI assays were performed on blood samples collected onto filter paper as described by Riddoch et al., (1996) or as described by Raidal et al., (1993a) when submitted as
plasma or serum. Confirmation of HA results by inhibition of HA activity with BFDV-specific antibody (Raidal et al., 1993a); filtration of samples through 0.22 µm filters; and or parallel testing with BFDV-insensitive galah erythrocytes was carried out as necessary whenever there was a discrepancy between HA and PCR test results. A comparative analysis of the data was performed using chi-squared tests for proportions using SPSS 4.0.

In an attempt to identify serotypes of BFDV, HA cross-reactivity was assessed by performing HI assays on 8 different BFDV isolates obtained from 2 rainbow lorikeets (Trichoglossus haematodus), a musk lorikeet (Glossopsitta concinna), a red lory (Eos bornea), 2 swift parrots (Lathamus discolor), 2 cockatiels (Nymphicus hollandicus) and a scarlet chested parrot (Neophema splendida) with blood samples containing known HI antibody titres (> 320 HIU/50 µL) obtained from 8 different psittacine species [2 short-billed corellas (Cacatua sanguinea), a sulphur crested cockatoo, 2 rainbow lorikeets, 1 red lory and 1 galah-corella hybrid].

Preparation and purification of DNA from feather and blood samples
DNA was extracted from feather tissues using modified Taberlat and Bouvet (1991) and Morin et al., (1994) methods as previously described by Ypelaar et al., (1999). DNA was extracted from the blood using the QIAamp DNA blood mini kit (QIAGEN).

Limit of detection of BFDV by HA and PCR assays
To determine the limit of detection of the HA and PCR assays serial 1:10 dilutions were prepared of an initial 10% (w/v) suspension made from the diseased feathers obtained from a long billed corella (Cacatua pastinator) with chronic PBFD and tested each dilution as described above for HA and below (Ypelaar et al., 1999) for PCR.

Amplification and analysis BFDV
PCR assay was performed as described by Ypelaar et al., (1999) with forward primer 5’-AACCCTACAGACGGCGAG-3 and reverse primer sequences 5’-GTCACAGTCCTGCTTGTACC-3, used to amplify a segment of BFDV ORF V1. All PCR products generated were visualised by agarose gel electrophoresis and a positive result was determined visually. Selected PCR amplicons of interest for DNA sequencing were purified from the agarose using the QIAquick Gel Extraction Kit (QIAGEN) and were ligated into pCR2.1 vector (Invitrogen) according to manufacturer’s protocol. The ABI Prism™ Dye Terminator Cycle Sequencing Kit (Applied Biosystem) was used according to manufacturer’s protocols except reaction volumes were halved to 10 µL and the annealing temperature was raised to 58°C. Sequence information was determined using the Applied Biosystem 3730 DNA Analyzer.

DNA sequencing of BFDV isolates from swift parrots and lorikeets
The generated BFDV ORF V1 sequence was edited and assembled using SeqEd version1.0.3 (Applied Biosystems) with corrections made on base-pair differences based on the chromatograms. All sequences were analysed using a range of programs provided by the Australian National Genomic Information Service (ANGIS), and National Center for Biotechnology Information (NCBI).

Edited sequence was analysed using the BLASTN and BLASTP programmes (Altschul et al., 1997) at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and compared with other previously published BFDV ORF-V1 data. The circovirus ORF-V1 nucleotide and amino acid sequences were aligned using programme Clustalx 1.8 (Thomson et al., 1997). Phylogenetic analyses were preformed using the neighbour-joining (NJ), maximum parsimony (MP) and maximum-likelihood
(ML) procedures with PAUP 4.0b2 and the Tajima–Nei model for distance estimation. A phylogenetic tree rooted to canary circovirus (Phenix et al., 2001) was constructed using the TreeCon 1.3b program with 1000 bootstrap cycles (Felsenstein 1985).

RESULTS

HA and PCR assays limit of detection
HA titres obtained from serial tenfold dilutions of feathers obtained from a corella with PBFD were >40,960, 25,600, 32,000, 40,000, <200,000 and <2,000,000 HAU/50 µL, respectively. A positive PCR result was obtained for the 1:10, 1:100 and 1:1000 but not in the 1:100,000 or 1:1,000,000 dilutions on the same material.

Comparison of HA, HI and feather PCR assay for the detection of BFDV
A total of 623 diagnostic accessions were received but not all accessions provided appropriate samples for all 3 tests. Of 621 feather samples received 143 (23%) were PCR positive. There was a strong agreement between the PCR and HA tests (kappa = 0.757 P<0.0001) and of the 143 feather samples 132 were also tested by both HA and 88 (66.7%) were also positive with HA titres ranging from 1:80 to 1:40,960 (mean log2 10.4 ± 2.6 HAU/50 µL) and 44 were HA negative but PCR positive. Of the remaining feather samples that were PCR negative 6 were initially positive by HA with titres up to 1:320 but the false-HA in these samples was not inhibited by anti-BFDV anti-sera and was removed by filtration through a 0.22 µm filter which indicated that BFDV was not the cause of the HA in the sample. Suspected false positive PCR results were obtained on a batch of feather samples from 4 clinically normal birds that were subsequently PCR negative on repeat retesting and a first-round false negative PCR result was detected in one bird that had a clinical description of PBFD and a very high feather HA titre.

There was a poor agreement between the PCR and HI test results (kappa = -0.193 P<0.0001) with PCR negative birds 12.7 (95% confidence interval = 3.1 to 52.6) times more likely to be HI positive than PCR birds. Of the 143 birds that were feather PCR positive only 2 had detectable HI antibody and these birds were also feather HA negative suggesting that they were developing immunity to recent infection. Otherwise HI antibody was detected in a total of 78 of 511 (15.3%) blood samples that were tested and HI titres ranged from 20 to 5,120 HIU/50 µL. All birds with HI antibody were negative on feather HA testing (kappa = -0.178 P<0.0001). No evidence of any antigenic serotypes was detected by HI cross-reactivity studies using feather and blood samples from different individual psittacine bird species.

In the peach face lovebird flock 47 out of 56 (83.9%) birds were PCR positive on blood samples but only 10 of these blood PCR positive birds (17.9% prevalence) were also PCR positive on feather samples. No bird was PCR feather positive without being PCR blood positive. Of the 10 blood PCR positive birds 5 had detectable feather HA titres ranging from 20 to 40,960 HAU/50 µL. Five birds that were PCR positive on both blood and feathers had no detectable feather HA. The 56 birds had a low seroprevalence (16%) and of the 9 birds that had detectable HI antibody titres (ranging from 20 to 320 HIU/50 µL) none were feather HA or PCR positive but 6 were PCR positive on blood samples.

Phylogenetic analysis of BFDV isolates
In this study, the ORF-V1 sequences from 9 new isolates of BFDV were determined with samples from 5 rainbow lorikeets (Trichoglossus haematodus), a red-collared lorikeet (T. haematodus rubritorquis), a musk lorikeet and 2 swift parrots. Pairwise analysis of the sequence analysis demonstrated that the isolates were 88-99%, at the nucleotide, and 87-98%, at amino acid (aa),
similar to each other and other published isolates. Phylogenetic tree analysis demonstrated a clustering of all the lorikeet isolates and one of the swift parrot isolates with all other BFDV isolates obtained from Loriidae species. The second swift parrot isolate was a unique genotype closely related to the Loriid group. To validate the sequences of the two swift parrot isolates DNA extraction, cloning and sequencing was repeated. The swift parrots were young wild birds that died with acute PBFD shortly after being taken by National Parks rangers from a nest on Bruny Island (near Tasmania).

**DISCUSSION**

The results indicate that PCR testing is more sensitive and specific than HA for detecting BFDV in feathers, because it detected evidence of viral DNA in feather suspensions that were HA negative (< 20 HAU/50µL), even though the analytical limit of detection of BFDV by HA was better (1 log₁₀) than PCR. This discrepancy is probably due to the enhanced process of extracting viral DNA from infected feather material in the absence of completed viral replication and release in feather dander. HA testing does provide a quantifiable indication of virus excretion and when used in conjunction with PCR provides a valuable internal control mechanism for the interpretation of results. From a diagnostic view point it is advantageous to know both results.

The presence of HI antibody in blood samples was inversely related to the presence of feather HA excretion and, except for one result, was also highly correlated with a negative feather PCR result. When used together all 3 tests can provide useful information about the BFDV-infection and immune status of a bird. Feather HA titres in excess of 640 HAU/50 µl, particularly in cockatoos (Raidal, *et al.*, 1993a), are highly correlated with the presence of chronic disease and have been used to confirm a clinical diagnosis of PBFD. Problems with non-specific HA reactions have not previously been reported in feather samples but are relatively common in faecal samples (Raidal *et al.*, 1993a). Confirmation of results by inhibition of the HA activity with BFDV-specific antibody is therefore recommended and should form part of a standard operating procedure. Parallel tests using BFDV-sensitive and BFDV-insensitive erythrocytes can also be used to ensure that the observed haemagglutination is specific and not due to other antigens (Raidal and Cross, 1994a).

The HI test has been used for seroepidemiological studies of BFDV infection in wild and captive birds (Raidal *et al.*, 1993b; Raidal and Cross 1994) and the presence of HI antibody titres is a strong negative predictive indicator for PBFD (Raidal *et al.*, 1993a; Ritchie *et al.*, 1991) but birds with active or latent BFDV infection may have low anti-BFDV HI titres that wax and wane. The non-detectable and low HI titres that occur in PBFD-affected birds may be explained by the severe damage that occurs to the bursa and thymus and or by the apparently persistent infections that occur in macrophages (Latimer *et al.*, 1991).

Interpretation of any BFDV infection diagnostic testing regime must consider the signalment, clinical signs and history of the bird and its environment. HA and HI assays are quantitative and provide valuable laboratory information that can influence clinical decisions but sources of suitable erythrocytes can be limited and differences in the agglutinating ability of erythrocytes obtained from different individuals of the same species have been reported (Sanada and Sanada, 2000). However, this is insufficient reason alone to discount HA as a diagnostic assay. As in any diagnostic assay, standardised procedures and appropriate internal controls should be used to provide reliable and valid results. Nevertheless there is a need to develop other methods for quantifying BFDV excretion in feathers and faeces because such information can be very important for guiding diagnostic judgements. Real time PCR assays for BFDV infection may provide this information (Raue *et al.*, 2004) but such techniques do so by detecting viral DNA and not antigen and their interpretation, from a clinical perspective, may not necessarily be any better than
conventional non-quantitative PCR methods.

There has been only one report of the development of a direct ELISA for detecting anti-BFDV antibodies in psittacine bird sera (Johne et al., 2004) but this method of testing has yet to be validated with a large number of samples from birds with known health status. HI assay is likely to remain the gold standard for anti-BFDV antibody detection for several reasons. The main advantage of the HI antibody detection system is that a secondary antibody directed against psittacine IgY is not required as is necessary in a direct ELISA. Johne et al., (2004) used a truncated recombinant capsid protein as the antigen in their ELISA and only 11 serum samples from 7 psittacine bird species were tested and the secondary antibody was raised against IgY from an African grey parrot. There have been only limited studies of the cross reactivity of anti-psittacine IgY antibody preparations so one could never be certain if serum from a rare species that tested negative was truly negative or if the secondary antibody failed to recognise immunoglobulin from that particular species. Within the Cacatuidae there are 6 genera including 21 species and within the Psittacidae there are 78 genera including 332 species. This present paper and others have shown that HI testing is suitable for detecting anti-BFDV antibodies in sera from a large proportion of these 353 species. The use of a truncated recombinant protein in an ELISA might also limit the assays specificity. Until these issues are resolved HI will probably continue to be the most reliable test for detecting BFDV antibodies.

The high blood PCR prevalence (83.9%) and low seroprevalence (16%) detected in the flock of *A. roseicollis* could be explained by the flock being recently infected following mixing of birds from different sources at the pet shop. Seroprevalences have been shown to be much higher in endemically infected flocks of *Agapornis* sp. (62%) and cockatoos (41-94%), (Raidal and Cross 1994b). Alternatively, there may be a high prevalence of latent or chronic carrier BFDV infections in *Agapornis* sp. Nevertheless, our observations that PCR can be more sensitive with blood versus feather samples is in contrast to that of Hess et al., (2004) who found a much higher prevalence of BFDV DNA in feather samples collected from budgerigars (*Melopsittacus undulatus*) even though there was poor correlation between PCR results and the presence or absence of feather lesions. Perhaps the reason for the higher prevalence of BFDV DNA in lovebirds compared to budgerigars could be explained by biological or immunological factors of the host species.

It is also important to consider that PCR tests may vary in sensitivity and specificity between laboratories even when the same primers and optimisation methods are employed (East et al., 2004). Our PCR prevalence data are similar to those reported by Bert et al., (2005) but much lower compared to those reported by Rahaus, et al., (2003) who found a much higher prevalence (39%) of BFDV DNA in feather samples collected from 146 clinically normal psittacine birds and even non-psittacine birds in Germany. Non-specific amplification of other avian circovirus amplicons was mooted as one possible reason for the latter observation but in our experience the primers designed by Ypelaar et al., (1999) do not amplify product from samples known to contain non-psittacine avian circoviruses. PCR assays for infectious agents have a theoretical high sensitivity and specificity but in practice they are rarely 100% sensitive or specific (East et al., 2004; Peter et al., 2000; Muller-Doblies 1998) and may even be only slightly more sensitive than conventional methods for virus detection (Mochizuki et al., 1993). Nested PCR assays can increase the sensitivity of an assay but the extra level of complexity can undo any gains in sensitivity or interfere with test specificity. False-negative PCR results may occur when inhibitors such as heparin (Holodniy et al., 1991) or biological materials in samples interfere with the assay (Konet et al. 2000) or with laboratory operator error. False positive results can occur with cross-contamination during sample collection or with laboratory handling and it is well accepted for other viruses that a positive PCR test result, on its own, is not a demonstration of active viral infection as non-replicating DNA may take up to 3 months to clear from blood (Lazizi et al., 1993) and it is for this reason that PCR-positive birds without clinical signs should be recommended for retesting.
PCR technology should be used together with, and not replace conventional diagnostic testing for PBFD (Cross, 1996). The data presented in this present paper indicates merit in having a 2 stage method for BFDV sample testing. In our experience, HA testing provides a valuable second method for identifying those birds that may be chronically affected and excreting large amounts of virus in feather dander versus those birds that may only be recently infected, and not shedding virus but mounting an effective immune reaction.

Ypelaar et al., (1999) found that consistent PCR results could only be achieved with primers designed to amplify ORF-V1 which codes for the Rep protein and thus more likely than the capsid protein to be genetically conserved. However, because of the diversity of BFDV genotypes PCR-based technologies may not detect all isolates even when conserved primers are used (Heath et al., 2004; Bassami et al., 2001; Ritchie 2003; Johne et al., 2004). This is another reason for having a 2 stage testing regime to capture isolates that may be genetically unique but still capable of causing haemagglutination. However we found no evidence of this possibility in our sample set and the 5 false positive HA reactors that we detected were cleared by filtering the sample through 0.22 µm filters and were not inhibited by anti-BFDV antibody.

There has been debate in the literature over the existence of a BFDV strain genetically adapted to lorikeets and parrots (Ritchie et al., 2003; Raue et al., 2004; de Kloet and de Kloet 2004; Heath et al., 2004) and the evolution of species-specific BFDV-genotypes such as cockatoo, budgerigar, lorikeet and lovebird lineages. This was the reason why we determined the DNA sequences of the isolates we obtained from lorikeets and the 2 swift parrot isolates. Swift parrots are an endangered species belonging to the Psittacidae family (Christidis et al., 1991, Christidis and Boles 1995) but are behaviourally and anatomically similar to lorikeets which justified studying the BFDV isolates obtained from these 2 birds. We compared the generated DNA sequence data with 36 previously described ORF-V1 BFDV sequences from psittacine birds from Australia, USA, UK, Germany, South Africa, Portugal, Austria and New Zealand. The results were genetically similar (86-97%) at the nucleotide level and, with the exception of one swift parrot isolate (isolate 3-SP-TS), our results are supportive of the clustering of BFDV isolates from lorikeets and lories into a Lorid genotype first proposed by Ritchie et al., (2003). There is a relatively high degree of genetic diversity in BFDV and, as more sequence data becomes available, the emergence of genotypes obtained from Psittacidae species is not unexpected given the greater number of extant bird species in this family. However, the biological significance of BFDV genotype clades is of unknown importance. Until transmission studies prove otherwise it must be continued to be assumed that all psittacine bird species are potentially susceptible to each genotype, indeed the putative high degree of recombination events within ORF-V1 supports this assumption (Heath et al., 2004).

There is evidence that recombination might contribute substantially more to genetic variation than genetic drift within ORF-V1 and this can result in inaccurate phylogenetic inferences (Heath et al., 2004). However, there is little proof of multiple BFDV isolate infections within psittacine hosts to permit such recombination events. The two sequences we obtained from swift parrots is good evidence that different isolates can at least naturally infect siblings within the same nest hollow. DNA sequence data from our two swift parrot isolates suggests that this species is naturally susceptible to both lorid and psittacid genotypes, which would be consistent with cross infection of BFDV between lorikeets and swift parrots. Swift parrots are a monotypic genus that probably evolved in the south east of Australia from a granivorous psittacid into a specialist nectarivorous bird before the more recent introduction of Trichoglossid lorikeets (Christidis et al., 1991). It competes closely for nectar and pollen as well as nesting sites with several lorikeet and parrot species (Gartell and Jones 2001) including, at Bruny Island, musk lorikeets, eastern rosellas (Platycercus eximius) and green rosellas (P. caledonicus). Swift parrots use different nest holes
each year according to the proximity of flowering trees (Dr Brett Gartrell pers comm). The wild swift parrot population currently consists of fewer than 1300 breeding pairs and is thought to be decreasing by more than 1% every year. Subclinical BFDV infections are well known in wild rainbow and scaly-breasted lorikeets in Australia which rarely develop chronically progressive lesions characteristic of PBFD in cockatoos but evidence that this is solely due to less virulent genotypes as suggested by Raue et al., (2004) rather than host defence factors is yet to be resolved. Such lorikeets pose a unique problem in that birds with clinical disease are frequently rescued and rehabilitated by wildlife carers in the eastern states of Australia which may promote the spread of BFDV carriers in the wild. Our results provide the first evidence that BFDV isolates derived from lorikeets may be able to infect other psittacine bird species.

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