STANDARDISED DIAGNOSTIC TESTS FOR BEAK AND FEATHER DISEASE VIRUS (BFDV)

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Non-technical Summary

The main aims of the project that Murdoch University undertook were to:

1. *Develop an ELISA for serological assay of BFDV antibodies in blood.*
2. *Compare this ELISA with HI testing already available and any other antibody testing method for BFDV that might also be developed during the course of the project.*
3. *Validation of the ELISA with blood of all psittacine bird species that might be tested.*
4. *Determine the sensitivities and specificities of the ELISA.*
5. *Determine the DNA genotypes of BFDV that require testing.*
6. *Determine the sensitivity of PCR testing on a variety of tissues (eg feather, blood) and environmental samples (eg nest hollow material).*
7. *Compare Haemagglutination (HA), Haemagglutination Inhibition (HI), ELISA and PCR tests to determine their relative sensitivities and specificities and which ones should be used in combination as a diagnostic panel.*

The deliverables under this project were to produce this document that reports against all of the agreed research milestones and at least one peer-reviewed research paper submitted for publication in a well-recognised and readily accessible scientific journal.

As outlined in the body of this document all of the objectives have been met and or exceeded. A blocking ELISA was developed that detects anti-BFDV antibody in any psittacine bird species. This assay was as sensitive and specific as existing and currently available serology assays and should supersede the HI assay as the gold standard for BFDV serology. A quantitative real-time PCR assay was also developed and shown to be more sensitive than existing PCR assays for detecting all of the known BFDV genotypes. All of the findings have been published or submitted for publication in internationally recognised peer-reviewed scientific journals.

Scientific Publications

Khalesi B, Bonne N.J., Stewart, M., Sharp M. & Raidal S.R. (2005). A comparison of haemagglutination (HA), haemagglutination inhibition (HI) and PCR for the detection of psittacine beak and feather disease virus (BFDV) infection and a comparison of isolates obtained from Loriids. *Journal of General Virology*. 86:3039-3046.

Stewart, M.E., Bonne, N. Shearer, P.L., Khalesi, B., Sharp, M. & Raidal., S.R. (2007). Baculovirus expression of beak and feather disease virus (BFDV) capsid protein capable of self assembly and haemagglutination. *Journal of Virological Methods.*41,(2),181-187

Shearer, P.L., Bonne, N., Clark, P., Sharp, M. & Raidal, S.R. (2008). Beak and feather disease virus (BFDV) infection in cockatiels (*Nymphicus hollandicus*). *Avian Pathology.* 37, (1), 75-81.

Bonne, N., Clark, P., Shearer, P. Raidal S.R. (2008). Filter paper dried biological samples and false positive PCR results. *Journal of Veterinary Diagnostic Investigation*. 20, 60-63.

Shearer, P.L., Bonne, N., Clark, P. & Raidal, S.R. (2008). Development and applications of a monoclonal antibody to a recombinant beak and feather disease virus (BFDV) capsid protein. *Journal of Virological Methods.* 147, (2), 206-212.

Bonne, N., Shearer, P.L., Clark, P. & Raidal, S.R. (2008). Assessment of recombinant beak and feather disease virus (BFDV) capsid protein as a vaccine for psittacine beak and feather disease (PBFD). *Submitted for publication*.

Shearer, P.L., Bonne, N., Clark, P. & Raidal, S.R. (2008). A blocking ELISA for the detection of antibodies to psittacine beak and feather disease virus (BFDV). *Submitted for publication*.

Shearer, P.L., Bonne, N., Clark, P. & Raidal, S.R. (2008). A universal quantitative, real-time polymerase chain assay for beak and feather disease virus (BFDV). *Submitted for publication.*

Technical Summary

Several diagnostic methods for the detection of beak and feather disease virus (BFDV) infection exist but there are few studies comparing the relative merits or sensitivity and specificity of each diagnostic test or how they can be used together to inform on the disease status of an individual bird or a flock. For this project we compared the results of PCR, haemagglutination (HA) and haemagglutination inhibition (HI) testing of diagnostic samples collected from 679 samples from a range of psittacine species suspected of being infected with BFDV. There was a strong agreement (kappa = 0.757 P <0.0001) between PCR and HA testing of feather samples and PCR negative birds were 12.7 times more likely to have HI antibody than PCR positive birds. False positive HA results with titres up to 1:320 were identified in 6 feather samples that were PCR negative because the haemagglutination detected in these samples was not inhibited by anti-BFDV anti-sera and was removed by filtration through a 0.22 μm filter. Similarly, 1 false negative PCR result was detected in a feather sample that had a high HA titre (>1:40,960) and 4 false positive PCR results were detected in a batch of 4 feather samples. Of 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. All birds with HI antibody were negative on feather HA testing. The assays confirmed BFDV infection in 2 endangered swift parrots (*Lathamus discolor*) and phylogenetic analysis of the sequence data generated from ORF-V1 of these isolates provide further evidence of BFDV genotypes clustering in parallel with the Loriidae, Cacatuidae and Psittacidae.

These results were used to develop a more sensitive and specific quantitative real-time PCR assay to permit the rapid detection of BFDV in diagnostic samples. Quantitative real-time PCR assays are extremely sensitive and have the advantages over standard PCR assays that they do not require post-reaction processing to visualise PCR products and they can quantify the amount of DNA present in a sample. Accordingly we developed a qPCR assay to amplify a conserved 81bp fragment of BFDV. A synthetic oligonucleotide was used to make standard curves for the quantitation of viral load in DNA extracts from the blood and feathers of 10 different species of birds which had previously tested BFDV-positive and samples from corellas experimentally infected with BFDV. The assay was very sensitive, with a detection limit of 50 copies/μL.

In order to develop an ELISA based serological assay for BFDV antibody the entire coding region of BFDV ORFC1 was expressed successfully in Sf9 insect cells using the baculovirus expression system to produce self-assembled virus-like particles (VLPs) which caused haemagglutination. The recombinant capsid protein reacted with anti-BFDV sera from naturally immune cockatoo and chickens experimentally inoculated with native BFDV in both western blots and haemagglutination inhibition (HI) assay and was used to develop a novel "blocking" (or "competitive") ELISA (bELISA) for the detection of anti-BFDV antibodies in psittacine sera in competition with a newly developed monoclonal antibody raised against the protein. The bELISA was then validated with 166 blood samples from eastern long-billed corellas (*Cacatua tenuiostris*) experimentally infected with BFDV and 82 blood samples from cockatiels known to be HI negative.

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Chapter 1. A comparison of haemagglutination (HA), haemagglutination inhibition (HI) and PCR for the detection of psittacine beak and feather disease virus (BFDV) infection

1.1 INTRODUCTION

Psittacine beak and feather disease (PBFD) is the most common viral disease of wild *Psittaciformes* (cockatoos, lorikeets & parrots) in Australasia and it has a world-wide distribution in captive birds. It is recognised as a threat for endangered psittacine birds in Australia, New Zealand and South Africa. In Africa, PBFD has been identified in captive *Agapornis* sp. (Kock, 1990) as well as wild cape parrots *Poicephalus robustus* (Heath *et al.* 2004), although these outbreaks may be due to the release of aviary birds. The disease is also widespread in captive collections of psittacine species in Europe and the USA.

The causative agent, BFDV, has a circular ssDNA ambisense genome (Bassami *et al.,* 1998; Niagro *et al.,* 1998) with two major open reading frames (ORF). ORF V1 encodes for the replication-associated protein (Rep) and ORF C1 encodes for the capsid protein. 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 & 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 & Sanada, 2000) but there have been no studies comparing the value of HA and HI testing relative to PCR. We decided to compare the three tests on feather and blood samples sent to us for routine diagnostic testing. We also sequenced PCR products from isolates obtained from Loriids to further investigate the current debate over the emergence of genetically adapted strains in lorikeets, parrots and cockatoos (Ritchie *et al.,* 2003; Raue *et al.,* 2004; de Kloet and de Kloet 2004; Heath, *et al.,* 2004).

1.2 MATERIALS AND METHODS

1.2.1 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. Although, 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.

1.2.2 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].

1.2.3 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).

1.2.4 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.

1.2.5 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′-GTCACAGTCCTCCTTGTACC-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.

1.2.6 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 performed 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).

1.3 RESULTS

1.3.1 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.

1.3.2 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 (Table 3). 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 $\log_2$ 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 (Table 3) 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 and a (Table 3). Table 4 provides the prevalences of BFDV feather excretion according to the major *Psittaciforme* groups.

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 (Table 5). 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.

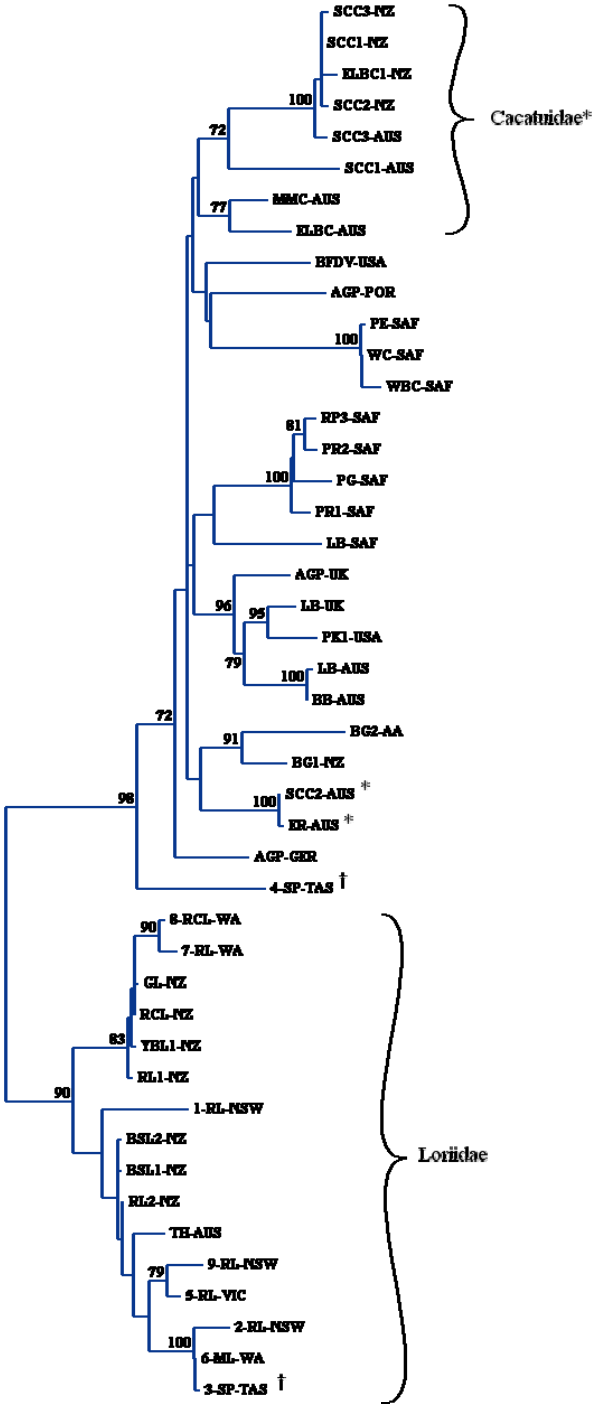

Figure 1 Neighbour joining tree of 45 BFDV ORFV1 nucleotide sequences rooted to the Rep gene of canary circovirus. Numbers at the nodes indicates percentage bootstrap support. Putative Loriid and Cockatoo clades are shown with outlying cockatoo* and swift parrot† isolates highlighted.

1.3.3 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. The details of these and reference isolates with their GenBank Accession numbers are summarized in Tables 1 and 2. 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 (Figure 1). 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).

Table 1. GenBank accession numbers for ORF-V1 DNA sequence data generated from 9 BFDV-infected lorikeets and swift parrots. Feather HA and blood HI titres are shown.

Isolate	Species	HA	HI	GenBank Accession No.
1-RL-NSW	Rainbow lorikeet Trichoglossus haematodus	1,280	< 20	DQ016388
2-RL-NSW	Rainbow lorikeet	40,960	< 20	DQ016389
3-SP-TAS	Swift parrot Lathamus discolor	40,960	< 20	DQ016390
4-SP-TAS	Swift parrot	40,960	< 20	DQ016391
5-RL-VIC	Rainbow lorikeet	40,960	< 20	DQ016392
6-ML-WA	Musk lorikeet Glossopsitta concinna	20,480	< 20	DQ016393
7-RL-WA	Rainbow lorikeet	5,120	< 20	DQ016394
8-RCL-WA	Red collared lorikeet T. haematodus rubritorquis	80	< 20	DQ016395
9-RL-NSW	Rainbow lorikeet	<20	< 20	DQ016396

Table 2. Circovirus reference sequences from psittacine and non-psittacine hosts used in this study.

Code	GenBank Accession No.	*Host species* (common name)
BB-AUS	AF311295	*Psephotus haematogaster* (Blue bonnet parrot)
LB-AUS	AF311296	*Agapornis roseicollis* (Peach faced lovebird)
ELBC-AUS	AF311297	*Cacatua tenuirostris* (Long billed corella)
ER-AUS	AF311298	*Eolophus roseicapillus* (Galah)
TH-AUS	AF311299	*Trichoglossus haematodus* (Rainbow lorikeet)
MMC-AUS	AF311300	*Cacatua leadbeateri* (Major Mitchell's cockatoo)
SCC1-AUS	AF311301	*Cacatua galerita* (Sulphur crested cockatoo)
SCC2-AUS	AF311302	*C. galerita*
SCC3-AUS	AF080560	*C. galerita*
BFDV-USA	AF071878	Pooled virus
PK1-USA	AY521234	*Psittacula krameri* (Indian ringneck)
LB-UK	AY521235	*A. roseicollis*
AGP-UK	AY521238	*Psittacus erithacus* (African grey parrot)
AGP-GER	AY521237	*P. erithacus*
LB-SAF	AY450442	*Agapornis nigrigenis* (Black-cheeked love bird)
PG-SAF	AY450441	*Poicephalus gulielmi* (Jardine's parrot)
WBC-SAF	AY450434	*Pionites leucogaster* (White-bellied caique)
PE-SAF	AY450435	*P. erithacus*
WC-SAF	AY450436	*Cacatua alba* (Umbrella cockatoo)
PR1-SAF	AY450437	*Poicephalus robustus* (Cape parrot)
PR2-SAF	AY450438	*P. robustus*
PR3-SAF	AY450439	*Poicephalus rueppellii* (Rüppell's parrot)
AGP-POR	AY521236	*P. erithacus*
SCC1-NZ	AY148285	*C. galerita*
SCC2-NZ	AY148286	*C. galerita*
SCC3-NZ	AY148287	*C. galerita*
LC1-NZ	AY148289	*C. tenuirostris*
RCL-NZ	AY148291	*T. haematodus rubritorquis* (Red-collared lorikeet)
YBL-NZ	AY148292	*Lorius chlorocercus* (Yellow-bibbed lory)
RL1-NZ	AY148293	*T. haematodus*
BSL1-NZ	AY148296	*Eos reticulata* (Blue-streaked lory)
BSL2-NZ	AY148297	*Eos reticulata*
GL-NZ	AY148298	*Psitteuteles goldiei* (Goldie's lorikeet)
RL2-NZ	AY148300	*T. haematodus*
BG1-NZ	AY148301	*Melopsittacus undulatus* (budgerigar)
BG2-AA	AJ605577	*M. undulatus*
CaCV	AJ301633	*Serinus canaria* (Canary)

Table 3. Test results obtained from diagnostic samples submitted from 623 psittacine birds showing the numbers of birds in each group and false negative and false positive results detected (italics).

No. birds	HI (blood)	HA (feathers)	PCR (Feathers)
6*	< 1:20	*1:20 – 1:320*	Negative
4†	< 1:20	< 1:20	*Positive*
1‡	<1:20	1:40,960	*Negative*
87	< 1:20	1:80 – 1:40,960	+
44	< 1:20	< 1:20	+
9	ND	ND	+
2	1:20 – 1:320	< 1:20	+
78	1:20 – 1:5,120	< 1:20	Negative
392§	< 1:20	< 1:20	Negative

* False positive HA titres (italicized) present in feathers from 5 samples that were PCR negative.
† False positive PCR results that on subsequent repeat retesting were PCR negative. These samples were 4 consecutive results in a batch suggesting they were probably due to laboratory contamination.
‡ False negative PCR result that on subsequent retesting was PCR positive.
§ In some of these cases not all 3 tests were done due to inappropriate or insufficient sample submission.

Table 4. Prevalence of BFDV shedding in feathers based on haemagglutination and or PCR testing of samples collected from 623 birds of various Families and genera.

Species	Total Number	No. BFDV positive (%)
Cacatuidae	282	87 (30.9 %)
Cacatua & Eolophus spp.	239	82 (34.3%)
Cockatiel (*Nymphicus hollandicus*)	12	1 (8.3 %)
Calyptorhynchus & Callocephalon spp.	31	4 (12.9%)
Loriidae	71	17 (23.9 %)
Lorikeets	58	15 (25.9 %)
Lories	13	2 (15.4 %)
Psittacidae	241	32 (13.3%)
Eclectus spp.	87	8 (9.2%)
African spp.*	24	6 (25%)
South American spp. †	59	4 (6.8%)
Asiatic spp.‡	28	4 (16.7%)
Australasian species	43	10 (23%)
Unknown	29	7 (24.2%)

* Jardine's parrot (*Poicephalus gulielmi*), African grey (*Psittacus erithacus*) & *Agapornis* spp.

† Macaws (*Ara* spp.), Amazons, Conures (*Aratinga* spp.) & Quaker parrots (*Myiopsitta monachus*)

‡ Indian Ringneck (*Psittacula krameri*), Alexandrine (*P. eupatria*), Derbyan (*P. derbiana*) & Moustached parrots (*P. alexandri*)

Table 5. Test results obtained from 56 peach faced lovebirds at a pet shop showing the numbers of birds in each group.

No. birds	HI (blood)	HA (feathers)	PCR (Blood)	PCR (Feathers)
5*	< 1:20	1:20 – 1:40,960	+	+
5	< 1:20	< 1:20	+	+
31	< 1:20	< 1:20	+	Negative
6	1:20 – 1:320	< 1:20	+	Negative
3	1:40 – 1:320	< 1:20	Negative	Negative
6	< 1:20	< 1:20	Negative	Negative

* Clinical examination of these five birds demonstrated feather lesions consistent with PBFD.

1.4 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 $\log_{10}$) 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 & Cross, 1994a).

The HI test has been used for seroepidemiological studies of BFDV infection in wild and captive birds (Raidal *et al.,* 1993b; Raidal & 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 & 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* (Table 5) 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 after 3 months (Dahlhausen & Radabaugh, 1993).

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 & 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 & 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 shown in an inferred phylogenetic tree (Fig. 1) rooted to canary circovirus are supportive of the clustering of BFDV isolates from lorikeets and lories into a Loriid 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 loriid 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 defense 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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Chapter 2. Expression of an antigenic beak and feather disease virus capsid protein using a baculovirus expression system

2.1 Introduction

The causative agent, BFDV, has a circular ssDNA ambisense genome (Bassami *et al.,* 1998) with two major open reading frames (ORF). ORF V1 encodes for a putative replication-associated protein (Rep) and ORF C1 encodes for a single putative capsid protein. Attempts at culturing BFDV in numerous culture systems have been unsuccessful. Thus current research on the virus and the epidemiology of infection has mostly been concerned with DNA-based detection assays and its genetic variation. The use of live birds to produce the antigen for BFDV serological assays is expensive, ethically questionable and considerably time consuming because it relies on the extraction of BFDV from the feathers of affected psittacine birds which can also create problems of unreliable antigen supply and host protein contamination. The latter can interfere with many types of serological assays as can antigenic variation but fortunately there appears to be no evidence of different BFDV serotypes (Khalesi *et al.,* 2005).

It has long been shown that virus structural proteins can self-assemble producing virus-like particles that imitate native virus morphology and immunological properties. The aim of this project was to express recombinant BFDV capsid protein, demonstrate that the protein self-assemble to produce VLPs, verify that the determinant for HA are located within the capsid protein and assess its ability to react with BFDV specific antibodies and ability to induce an antibody response. FDV ORF C1 has been predicted to encode the capsid protein, the major structural protein of BFDV which has a molecular mass of 31 kDa. This is the immunodominant antigen in the virus and it is associated with haemagglutinating activity, induction of haemagglutination-inhibition antibodies and induction of a protective immune response.

2.2 Materials and methods

2.2.1 Primers and PCR

An Australian BFDV isolate by Bassami *et al*. (1998) that had previously been sequenced (Genbank accession number AF080560) and characterised and found to be most similar to other isolates was used as a template in construction of recombinant plasmids.

Oligonucleotide primers were designed to amplify the entire coding sequence of ORF C1 (cap gene) (Table 5.1). Engineered into the 5' end of each primer was the recognition site for the restriction enzyme *Eco*RI *and Sal*I. This facilitated construction of the recombinant

pFastBac™ plasmid. The primer pair BFDV ORF C1 forward and BFDV ORF C1 reverse was used to amplify a 743 nt subfragment.

Table. Primers used for the amplification of genes of interest from BFDV ORF C1 template DNA. All primers contain the coding sequence for the restriction enzyme *Eco*RI, *Sal*I at the 5' end.

NAME	SEQUENCE (5' 3')	NUCLEOTIDE TARGET IN GENOME	ANNEALING TEMPERATURE (°C)
FORWARD PRIMER	GAAATGTGGGGCACCTCTAACTGCG GTCTAAGTGCTGGGATTGTTAGG	1-22	60
REVERSE PRIMER		725-745	60

PCR reactions were performed in 25 µL volumes containing 2 mM $MgCl_2$, 2 mM of each deoxynucleotide triphosphate and 0.5 U of AmpliTaq DNA polymerase (Perkin Elmer) made up to volume with PCR buffer (PerkinElmer). The reaction was carried out in a Cetrius 2400 Thermocycler (PerkinElmer) using an initial denaturing step at 96^{o}C for 5 min, 32 cycles of denaturing 96^{o}C for 0.5 min, annealing at 60°C for 0.5 min and extension at 72^{o}C for 1.5 min, with a final extension at 72^{o}C for 10 min. This was followed by a final extension step at 72°C for 10 min to provide better efficiency of 3' deoxyadenosine triphosphate addition to PCR products, useful in TA cloning.

2.2.2 Escherichia coli strains

The strains and genotypes of *E. coli* utilised for cloning and expression of baculovirus recombinant proteins were *E. coli* TOP10 (F^{-} mcrA Δ (mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL (Str^{R}) endA1 nupG), *E. coli* TOP10F' (F' {lacI^{q} Tn10(Tet^{R})} mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 deoR araD139 Δ (ara-leu)7697 galU galK rpsL(Str^{R}) endA1 nupG) supplied in the TOPO TA Cloning® kit (Invitrogen) and *E. coli* DH10Bac™ (F^{-} Δ mcrA (mrr-hsdRMS-mcrBC) ϕ80dlacZ M15 Δ lacX74 endA1recA1 deoR (ara, leu) 7697araD139 galU galK nupG rpsLλ^{-}/bMON14272/pMON7124) supplied in the Bac-to-Bac® Baculovirus Expression System (Invitrogen).

2.2.3 Preparation of plasmid DNA

Plasmids pFastBac™ HT A and pCR® 2.1-TOPO® were purified using a plasmid DNA mini-prep procedure modified from Morelle (1991). Briefly, a 10 mL volume of overnight culture in LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 100 µg/mL ampicillin (Sigma), shaken at 250 rpm and incubated at 37°C. Approximately 3 mL of culture broth was centrifuged (21,000 x g, 30 s) to pellet the bacteria which were then

resuspended in 200 µL of solution I (25 mM Tris-HCl, 10 mM EDTA and 20 µg/mL RNAse A; pH 8.0) and an additional 200 µL of solution II (1% [w/v] SDS and 0.2 M NaOH) to lyse the bacterial cells. The mixture was inverted several times for complete cell lysis before the addition of 200 µL solution III (3 M potassium acetate; pH 4.8) to neutralise the pH and precipitate proteins. The precipitated protein was pelleted by centrifugation (21,000 x g, 20 min). The supernatant was mixed with 600 µL of 100% isopropanol to precipitate all nucleic acids, and centrifuged (21,000 x g, 20 min). The pellet was blotted onto paper towels to remove excess liquid and 500 µL of 70% (v/v) ethanol was added to wash salts away. The tube was then centrifuged (21,000 x g, 10 min). The pellet was dried under a vacuum in a Savant Rotary SpeedVac for 10 min and subsequently resuspended in 50 µL dH2O. Plasmid DNA preparations were subsequently used for restriction endonuclease digestion and PCR analysis.

2.2.4 Restriction endonuclease digests of plasmids

Restriction digests were performed in a total volume of 20 µL containing 2 µL 10X reaction buffer, 5 µL DNA template (approximately 800 g plasmid DNA), 1 µL restriction endonuclease (10 U) and 12 µL dH2O. The digest reactions were incubated for at least 2 h at 37°C. Where incubations exceeded this time, 0.5 µL (5 U) of enzyme was used and the residual volume replaced by an appropriate amount of dH2O.

2.2.5 Ethanol precipitation of DNA

To enable efficient precipitation of DNA, sodium acetate (pH 5.2) was added to the preparations to a final concentration of 1 M, then 2 volumes of 100% (v/v) ethanol. After incubation at 20°C for at least 1 h, the sample was centrifuged (21,000 x g, 15 min, 4°C), the supernatant removed and the pellet washed with 500 µL of 70% (v/v) ethanol and further centrifuged (14,000 x g, 5 min, 4°C). The supernatant was aspirated and excess supernatant removed by gentle tapping on a paper towel. The DNA pellet was dried under vacuum in a Savant Speed Vac Concentrator for 5 min at room temperature and then resuspended in double-distilled deionised water (ddH2O).

2.2.6 Agarose gel electrophoresis of DNA preparations

Agarose gel electrophoresis was performed using either Bio-Rad Mini Sub™ DNA cells or BioRad Wide Sub™ DNA cells (Bio-Rad). Tank and gel buffers consisted of TAE (50X TAE: 2 M Tris-Acetate, 0.05 M EDTA, pH 8.0) as described by Sambrook *et al*. (1989). Agarose gels consisted of 0.8% (w/v) electrophoresis-grade agarose (Progen) and 10 µg/mL ethidium bromide. Electrophoresis was performed at 90 V for approximately 45 min and the gels viewed under ultraviolet light with a transilluminator. Gels were photographed using the GelDoc system (Bio-Rad).

2.2.7 Extraction of DNA from agarose gels

The DNA band of interest was excised from the agarose gel and the volume estimated and the DNA recovered from the excised band using a Qiagen Gel Extraction kit (Qiagen) as described by the manufacturer. Briefly, approximately 3 volumes of QX1 buffer

(composition not supplied) were used to dissolve the agarose at 56°C for 10 min. An equivalent volume of 100% isopropanol to the volume of the gel excised was then added to the solution and the mixture allowed to equilibrate. The samples were then loaded onto a spin column (Qiagen) and centrifuged (21,000 x g, 1 min). Following a wash with 500 µL of QX1 buffer, the column was washed with a PE buffered solution containing ethanol (composition not supplied). The column was centrifuged and dried. DNA was eluted from the column with 30 µL dH2O for 1 min and then centrifuged (21,000 x g, 1 min). The resulting DNA solution was stored at -20°C until used.

2.2.8 Ligation of vector and insert DNA

Ligation reactions were typically performed in a total volume of 10 µL containing ligation buffer (New England Biolabs), a determined amount of linearised plasmid (typically 25 g), an appropriate amount of insert and 4 Weiss units of T4 DNA ligase (New England Biolabs) in sterile dH2O. The ligation reaction was incubated overnight (~15 h) at 14°C. The reaction was stopped by heat inactivation of the enzyme at 70°C for 10 min and then the mixture was cooled to room temperature. Ligation mixtures were stored at -20°C until used later for transformation.

2.2.9 Cloning PCR products into pCR® 2.1-TOPO®

A TOPO TA Cloning® kit (Invitrogen) was used as recommended by the manufacturer to clone PCR products into the plasmid vector. Briefly, unpurified PCR products were quantified relative to molecular weight standards and added to a TOPO® cloning reaction mixture with an insert: vector molar ratio of 3:1. The 6 µL TOPO® cloning reaction mix contained 1 µg pCR® 2.1-TOPO® plasmid DNA, an appropriate amount of PCR product, 1 µL salt solution and sterile water to make up the 6 µL reaction volume. The TOPO® cloning reaction was performed at 22°C for 5 min and then stored at -20°C until used for transformation.

2.2.10 Cloning DNA fragments into pFastBac™

Approximately 500 µg of pFastBac™ HT A (Invitrogen; Figure 5.2), a baculovirus expression vector containing an N-terminal hexahistidine (6 x His) tag, was digested with the restriction endonuclease *Eco*RI and *Sal*I in a 20 µL reaction as described above, then dephosphorylated to reduce re-ligated plasmid background and increase the possibility of ligation of vector with the DNA fragment to be cloned. Dephosphorylation of restriction endonuclease-digested vector was performed by treatment with 20 U calf intestinal alkaline phosphatase (CIAP; New England Biolabs) for 3 cycles of 15 min at 37°C followed by 15 min at 56°C. The dephosphorylated plasmid was then recovered by ethanol precipitation and quantified using a Dynaquant DNA fluorometer (Hoefer). Purified gel-extracted DNA to be cloned was quantified relative to DNA marker standards or by fluorometry. Ligation was performed by standard procedures outlined above using a 5:1 insert: vector molar ratio.

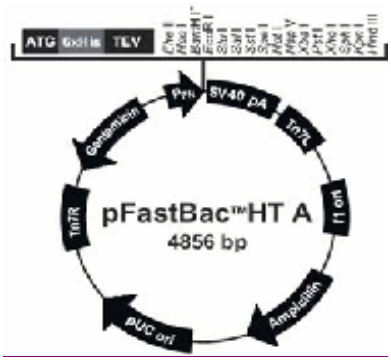

Figure 2 Vector map of pFastBac™ HT A.Polyhedrin promoter (PPH) allows efficient high level expression recombinant protein in insect cells. SV40 polyA signal permits efficient transcription termination and polyadenylation of mRNA. http://www.invitrogen.com

2.2.11 Transformation of pCR® 2.1-TOPO®

Transformation of competent *E. coli* TOP10F' cells with TOPO® cloning reaction mixture by the heat-shock method was performed as recommended by the manufacturer. Briefly, 2 µL of TOPO® cloning reaction mixture was added to 50 µL of One Shot® Chemically Competent TOP10F' *E. coli* cells (Invitrogen). Transformation reactions were incubated on ice for 30 min, heat shocked at 42°C for exactly 30 s and transferred back to ice. A 250 µL volume of SOC medium (Invitrogen) at room temperature was added to the transformation mixture and the tube shaken at 200 rpm at 37°C for 1 h. Fifty to 100 µL of the transformation mix was spread onto pre-warmed LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1.5% [w/v] agar supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin) containing X-Gal and IPTG, and grown overnight at 37°C. Resultant colonies were analysed by isolating the plasmid DNA, as described above, and digestion with appropriate restriction enzymes to ensure the plasmid contained the appropriately sized insert.

2.2.12 Transformation of pFastBac™ HT A into E. coli TOP10 cells

Transformation of competent *E. coli* TOP10 cells with pFastBac™ HT A was performed by a heat-shock method. A 3 µL volume of the ligation reaction mix was added to a 1.5 mL microcentrifuge tube containing 25 µL of One Shot® Chemically Competent TOP10 *E. coli* (Invitrogen). Cells were incubated on ice for 30 min and then heat-shocked for 30 s at 42°C. The tubes were placed on ice and 250 µL of SOC medium at room temperature was added. Tubes were shaken at 200 rpm at 37°C for 1 h. Fifty and 100 µL volumes of the transformed cells were spread on LB agar plates containing 100 µg/mL ampicillin. Plates were incubated overnight at 37°C. Resultant colonies were analysed by isolating the plasmid DNA, as described above, and digesting it with appropriate restriction endonucleases to ensure the plasmid contained the appropriate insert in the correct orientation for protein expression. Further characterisation of recombinant clones was performed by sequence analysis of the plasmid insert. Once a recombinant clone in the correct orientation and frame was identified, transformation into DH10Bac competent cells was performed.

2.2.13 Transformation of DH10Bac™ E. coli

Once the recombinant pFastBac™ construct had been generated, it was necessary to transform the purified plasmid DNA into DH10Bac™ *E. coli*. The *E. coli* DH10Bac™ strain contained a baculovirus shuttle vector (bacmid) with a mini-attTn7 target site and a helper plasmid. Once the pFastBac™ expression plasmid was transformed into the DH10Bac™ cells, transposition occurred between the mini-Tn7 element on the pFastBac™ vector and the mini-attTn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurred in the presence of transposition proteins supplied by the helper plasmid. Blue/white selection was then used to identify colonies containing the recombinant bacmid. For each transformation, 100 µL of MAX Efficiency® DH10Bac™ competent cells (GibcoBRL) was thawed on ice and transferred into a pre-chilled, 15 mL round-bottom polypropylene tube. Approximately 1 µg of purified pFastBac™ HT A plasmid DNA containing the gene of interest was added to the cells and mixed gently. The cells were incubated on ice for 30 min, then heat-shocked for 45 s at 42°C without shaking. The tubes were then immediately transferred to ice and chilled for 2 min. Following this, 900 µL of room temperature SOC medium was added and the tubes shaken (225 rpm) at 37°C for 4 hours. For each pFastBac™ transformation, 10-fold serial dilutions of the cells were prepared with SOC medium and 100 µL of each dilution was plated on an LB agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal (GibcoBRL), and 40 µg/mL IPTG. Plates were incubated for 48 h at 37°C and white colonies picked for analysis.

2.2.14 Analysis of recombinant bacmid DNA by PCR

Recombinant bacmid was recovered in the same way as plasmid DNA and then analysed by PCR for presence of the desired gene; the recombinant bacmid DNA was greater than 135 kb in size thereby making restriction endonuclease analysis difficult to perform. The bacmid contained M13 Forward (5' GTTTTCCCAGTCACGAC 3') and M13 Reverse (5' CAGGAAACAGCTATGAC 3') priming sites flanking the mini-attTn7 site within the lacZa-complementation region to facilitate PCR analysis and the recombinant bacmid DNA was amplified using M13 Forward and M13 Reverse primers and Taq DNA polymerase. Each PCR had a 50 µL reaction volume composed of 100 g recombinant bacmid DNA, 5 µL 10X PCR buffer, 1 µL 10 mM dNTP mix, 1.5 µL 50 mM $MgCl_2$, PCR primers (1.25 µL each, 10 µM stock), 0.5 µL Platinum® Taq polymerase (Invitrogen) and sterile water to make up the 50 µL volume. The thermal cycling profile were an initial denaturation step at 93°C for 3 min, followed by 25 to 34 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45sec and extension at 72°C for 5 min, followed by a final extension at 72°C for 7 min.

2.2.15 Transfection of insect cells

Once it was confirmed that the recombinant bacmid contained the gene of interest, transfection of insect cells was performed to produce recombinant baculovirus. In a 6-well or 35 mm tissue culture plate, Sf9 insect cells (9×10^5 cells per well of a 6-well plate) were seeded in 2 mL of Sf-900 II SFM (Invitrogen) containing antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin). Cells were allowed to attach at 27°C for at least 1 h. For each

transfection, 1 µg of purified bacmid DNA was mixed with 100 µL of Sf-900 II SFM and 6 µL of Cellfectin reagent (GibcoBRL). The mixture was incubated for 45 min at room temperature. While the DNA: lipid complexes were incubated, media was removed from the cells and they were washed once with 2 mL of Sf-900 II SFM. To each tube containing the DNA: lipid complexes, 0.8 mL of Sf-900 II SFM was added, mixed gently and added to one well of cells. Cells were incubated at 28°C for 5 h before the DNA: lipid complexes were removed and 2 mL Sf-900 II SFM containing antibiotics was added. Cells were incubated again at 28°C but this time in a humidified environment for 72 h or until a cytopathic effect (CPE) developed. Once the transfected cells showed an advanced CPE, the medium (containing virus) from each well was collected and transferred to a sterile 15 mL tube. The tubes were centrifuged (500 x g for 5 min) to remove cells and large debris, and the clarified supernatant was transferred to fresh 15 mL tubes.

2.2.16 Amplification of recombinant baculovirus

The viral stock recovered from the transfection was of low titre. To increase the titre, Sf9 cells were infected at a multiplicity of infection (MOI) ranging from 0.5-5. The MOI was defined as the number of infectious virus particles per cell. The infected cells were incubated with the inoculum at room temperature for 1 h to allow for attachment and the cells were then incubated for 48 h at 28ºC. The cell culture medium was collected and transferred to sterile tubes, centrifuged (500 x g for 5 min) to remove cells and large debris, and the clarified supernatant pooled and stored at 4ºC, protected from light.

2.2.17 Titration of recombinant baculovirus by plaque assay

The titre of infectious virus in the baculovirus stocks was determined by plaque assay. Approximately 1 x 10^6 Sf9 cells were seeded into each 35 mm well of a 6-well plate, then incubated at room temperature for at least 1 h to ensure cell attachment, then the medium was aspirated. Ten-fold serial dilutions of the virus preparation were prepared and 1 mL of each dilution added to wells and the monolayers incubated at room temperature for an additional 1 h. The virus inoculum was then aspirated and replaced with 2 mL of a plaquing overlay consisting of Sf-900 II SFM 1.3X (GibcoBRL) and sterile 4% (w/v) agarose at a ratio of 3:1. Plates were incubated at 28°C in a humidified incubator for 4 days, then the cells were stained by the addition of 2 mL of 0.03% (w/v) neutral red and incubated for 1 h at room temperature. Excess stain was aspirated and the plates were inverted and placed in the dark at room temperature until the number of visible plaques did not change for 2 consecutive days. To determine the titre as pfu (plaque forming units)/mL of the viral inoculum, the plaques were counted in wells containing 3-20 plaques (Figure 5.3).

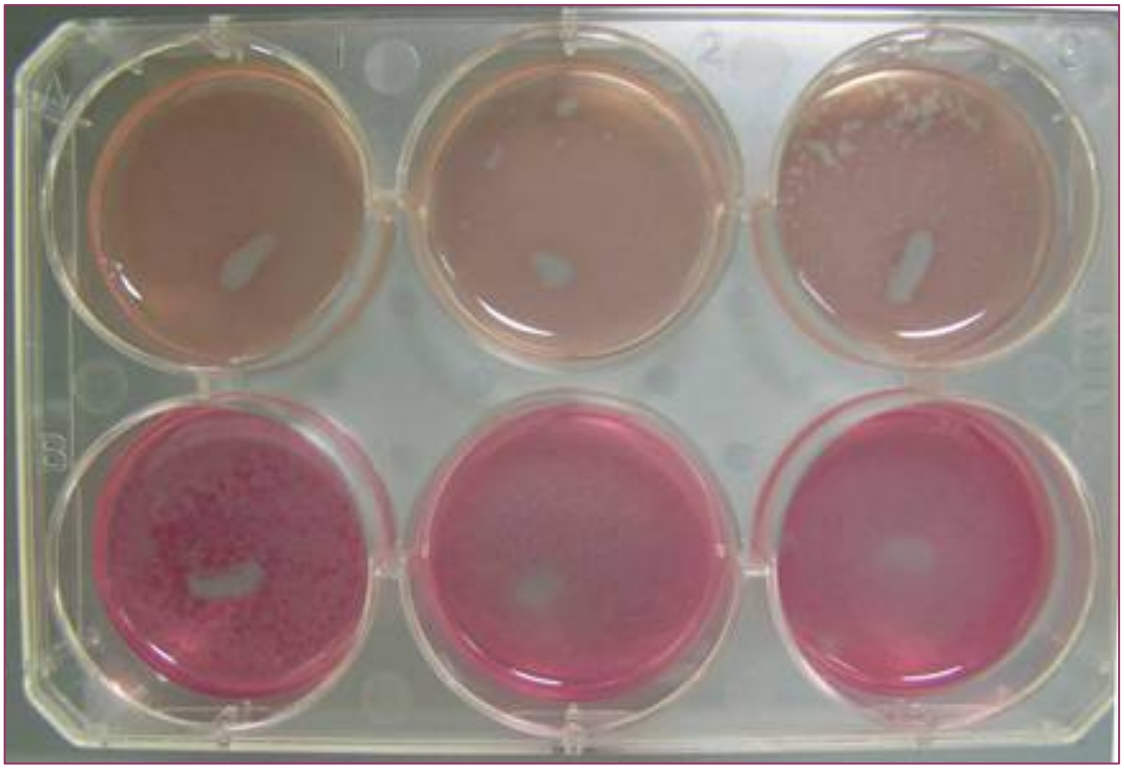

Figure 3. Baculovirus plaques generated by infection of Sf9 cells with baculovirus. Cells were stained with neutral red.

2.2.18 Expression of baculovirus proteins in insect cells

Once a pFastBac™ baculovirus stock was generated with a titre of 1 x 10^8 pfu/mL or greater, it was possible to infect insect cells and assay for expression of recombinant protein. Suspension cultures of Sf9 cells were grown in serum-free conditions in Sf-900 II SFM, and growth was monitored by counting viable cells in a haemocytometer using trypan blue exclusion. Once cultures reached a density of 2 x 10^6- 3 x 10^6 viable cells/mL they were infected with the recombinant baculovirus at an MOI of 5. Infected cells were monitored at regular intervals and harvested 48 h post-infection by centrifugation (500 x g for 10 min). The diameter insect cell and the size of nuclei increased over 24 hours cells ceased to grow and appeared granular (Figure 5.4).

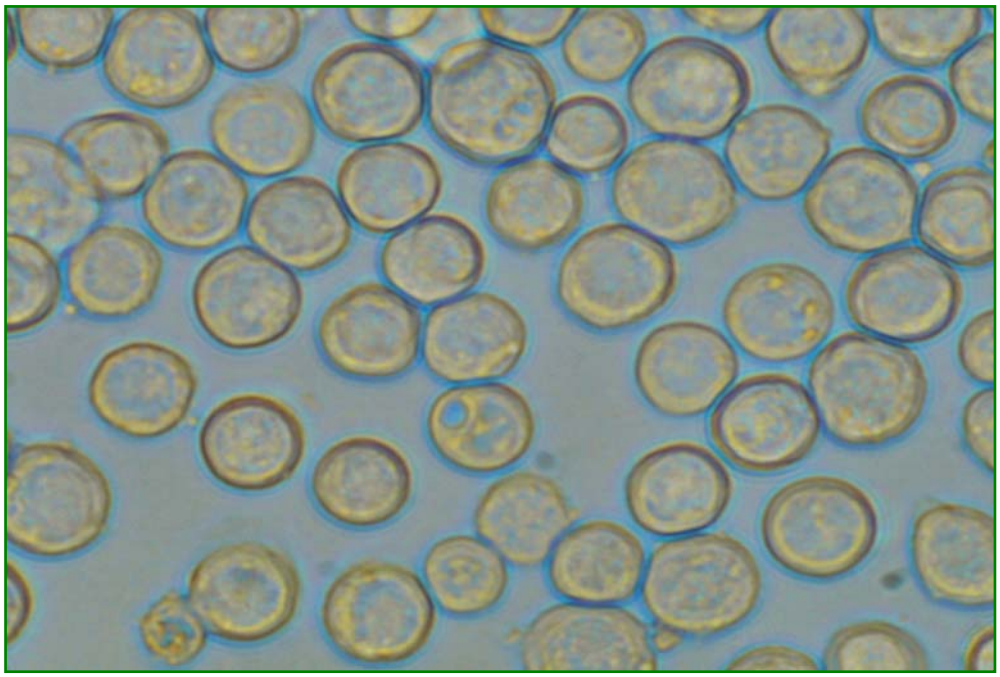

Figure 4. recombinant bacmid transfected infected sf9 cells under phase contrast light microscope.

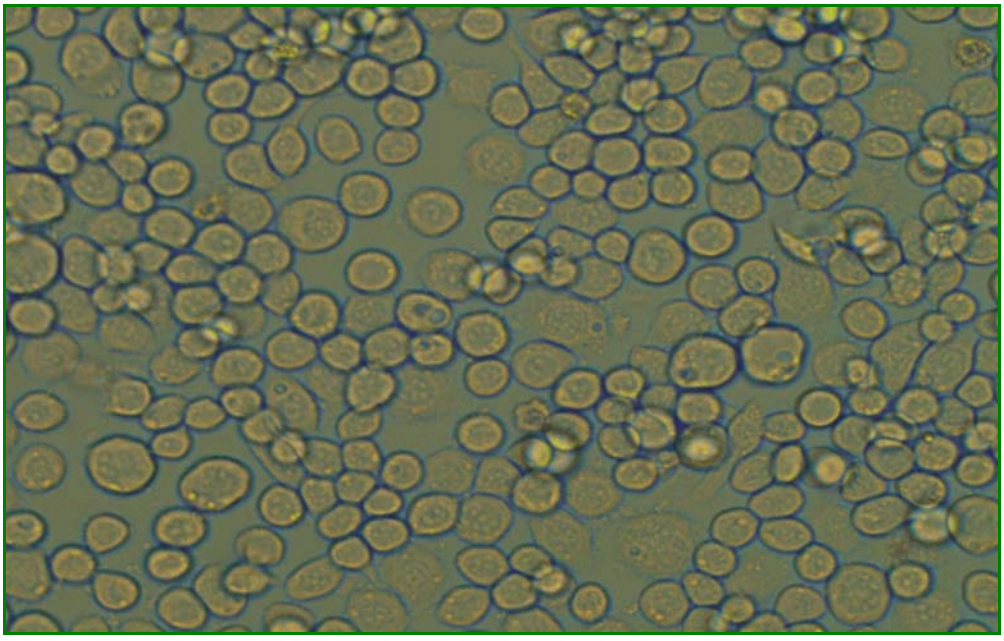

Figure 5. Uninfected sf9 insect cells.

2.2.19 Purification of recombinant BFDV ORF C1

Two methods of purification were developed to purified recombinant BFDV ORFC1 from the SF9 insect cell lysates; NiNTA affinity and caesium chloride ultra centrifugation. The infected Sf9 insect cells were harvest 72 hr post infection at low speed centrifugation (1500 x

g, 10 min), washed in 10 ml of PBS. The Sf9 insect cell pellet was suspended in Sf9 Cell Lysis Buffer (50 mM Tris-HCl pH 8.5, 100 mM KCl, 1 mM PMSF, 1% w/v Nonidet P-40, and 0.5% v/v β-mercaptoethanol) and lysed by incubated at 4°C for 1 h on a rocker followed by sonication. The sample was examined to ensure complete lysis had occurred prior to proceeding. The lysed sample was centrifuged at 10 000 x *g* for 10 min 4°C to remove cell debris and the supernatant transferred to Ni-NTA resin (QIAGEN) equilibrated in Sf9 cell lysis buffer and the recombinant proteins incubated with the resin overnight at 4°C on a rocker to allow efficient binding of the protein. The lysate was removed and the resin with the bound recombinant protein was washed three times in 10 resin volumes of Wash Buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 10 mM Imadazole). The recombinant BFDV ORFC1 protein was eluted from the Ni-NTA resin in 2 resin volumes of Elution Buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 300 mM Imadazole) at 4°C after 30 min incubation.

The recombinant recombinant BDFV ORFC1 protein was purified from the cell lysate by ultra centrifugation to determine if VLP were produced. The SF9 insect cell were harvest 72 h p.i and lysed as previously described. A sucrose buffer was initially used to purify VLPs from the debris and the cell lysate was layered onto a 40% (w/v) sucrose cushion and centrifuged at 28 000 rpm 2 hr (SW55Ti, Beckman) at 18°C. The supernatant was discarded and the pellet suspended in 1x PBS and the VLP purified according to the caesium chloride ultracentrifugation method for the BFDV (Ritchie *et al.,* 1989; Raidal and Cross 1994). The fraction containing the recombinant BFDV ORFC1 VLPs was determined by SDS-PAGE, western immuno-blot, electron microscopy and HI. The total protein concentration was determined by the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) used as a standard.

2.2.20 SDS Page and Western immunoblot

The protein preparations were initially analyses by denaturing SDS-PAGE and Western immuno-blot to enalble the size of the his-tagged recombinant protein to be determined.
Proteins samples were diluted in SDS-PAGE sample buffer (Laemmli, 1970) and separated by 12.5% denaturing SDS-PAGE gel (Laemmli, 1970), electrophoresed at 180 V for 1 h and stained with Coomassie brilliant blue (Bio-Rad) to visualise the proteins. For western immuno-blot, the proteins transferred to nitrocellulose membrane (BioRad) using a Transblot SD semidry transfer cell (BioRad) 30 min and 15 V with the semi dry apparatus. Membranes were incubated in blocking solution (1× Phosphate buffered saline, 0.05% v/v Tween-20, 5% w/v skim milk powder) for 1 hour at 37°C.

Naïve chicken and chicken anti-BFDV sera with a HI titre of 5,120 HIU was kindly provided by Dr Gary Cross, University of Sydney. The membrane was incubated with chicken-anti-BFDV sera at a dilution of 1:200. A series of cockatoo-anti-BFDV sera with HI titres ranging from 160 – 5120 HIU was provided by Dr Shane Raidal. The membrane was incubated with cockatoo-anti-BFDV sera at a dilution of 1:20 – 1:100. To enable detection all of the cockatoo-anti-BFDV sera bound to the recombinant protein the membranes were incubated with goat-anti-cockatoo sera (kindly supplied by Sarah Plant, Murdoch University) diluted to 1:200.

The recombinant protein was screened against a panel of psittacine species serum that had been previously determined HI positive and negative (1:10, 1:20 and 1:50), naïve polyclonal

chicken serum (1:200) and polyclonal chicken anti-BFDV serum (1:200) and mouse anti-His monoclonal (1:5000; Sigma). Serum was diluted in blocking solution and incubated with the nitrocellulose membrane for at least 2 h at RT. The psittacine serum had to be incubated with polyclonal goat anti-psittacine diluted in blocking solution for 2 h prior the incubation with a conjugated antibody for detection. The membranes were incubated with either HRP conjugated anti-goat IgG (1:2000; ICN), HRP-conjugated anti-chicken IgG (1:5000; ICN) and HRP-conjugated anti-mouse IgG (1:2500; ICN) and HRP-mediated detection was performed by addition of western development substrate (HRP development and H_2O_2; BioRad)

2.2.21 Quantitation of purified protein

Protein solutions were assayed in duplicate or triplicate using the BioRad Protein Assay Kit (BioRad). Three to 5 dilutions of a bovine serum albumin (BSA) protein standard ranging from 8-80 µg/mL were prepared as standards. A volume of 160 µL of each BSA standard, or a protein solution of unknown concentration was pipetted into a microtitre plate well. A 40 µL volume of dye reagent concentrate (BioRad) was added to each well, the plates were shaken on a microplate mixer, incubated at room temperature for at least 5 min, and then the absorbance measured at 595 nm using a microplate reader.

2.2.22 Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were visualised by 0.1% (w/v) sodium dodecyl sulphate, 12.5% (v/v) polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The samples were then loaded onto a prepared mini-gel and electrophoresed at 200 V for 45 min. Gels were stained with Coomassie blue and destained with destain solution (40% methanol, 10% acid acetic glacial in water), or subjected to Western immunoblotting. Destained gels were stored after they were dried with a vacuum gel dryer (BioRad).

2.2.23 Western immunoblotting

Detection of recombinant antigens by Western immunoblot analysis was performed using the procedure originally developed by Towbin *et al*. (1979). Unstained SDS-PAGE gels were transferred to 0.45 µm nitrocellulose membranes using a mini Trans-Blot apparatus (BioRad) and ice-cold transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol). Transfer was performed at 100 V for approximately 1 h at 4°C. The membrane was blocked overnight at 4°C with PBSTM and then washed in PBST. Primary antibody was added to the membrane at a dilution of 1:200 for chicken anti BFDV, or 1:5,000 for mouse anti-His IgG, and the membrane incubated at 37°C for 2 h. After 3 washes in PBST a horseradish peroxidase (HRP) conjugated secondary antibody was added to the membrane at a dilution of 1:2,500 for anti-mouse (goat anti-mouse IgG-HRP conjugate; Sigma), or 1:2,500 for chicken anti-BFDV (mouse anti-porcine IgG-HRP conjugate; ICN) and incubated at 37°C for 1 h. The membrane was then washed and allowed to react with 4-chloro-naphthol (4-CN) substrate (BioRad). The reaction was stopped by rinsing the membrane under distilled and deionised water and allowed to dry. For assay of field serum samples, purified recombinant fusion protein was applied to an SDS-PAGE gel with no wells, producing a continuous band of antigen across the gel. All Western immunoblot assays were performed using reference positive antisera on one strip from each batch of strips obtained from one nitrocellulose membrane.

2.2.24 Immunisation of chickens with recombinant protein

A volume of 250 µl purified baculovirus recombinant protein was mixed with 250 µl incomplete Freund's adjuvant using glass syringes and this was used to vaccinate 4 clinically normal chickens with 0.5 ml of recombinant BFDV C1 vaccine injected into pectoral muscle. Blood samples were collected onto filter paper (Whatman No.3) before injection, 7 day 22 and 36 days post vaccination. The first injection of chickens was done on day 7 and the first booster was on day 22. Antibody response was detected using the filter paper method and haemagglutination inhibition assay (HI).

2.2.25 Haemagglutination assay (HA) and Haemagglutination Inhibition (HI) Assay.

HA assays were performed using serial dilution of 2-fold, 3-fold, 5-fold, 10-fold and 100-fold dilutions on the purified BFDV ORFC1 protein and BFDV VLPs using galah (*Eolophus roseicapillus*) erythrocytes as described by Raidal *et al* (1993). The HA results were confirmed by inhibition of HA activity with BFDV-specific antibody (Raidal *et al.,* 1993). Negative controls including baculovirus expressed PCV-1 ORFC1 (a kind gift from Dr A Hughes), wild-type baculovirus, Sf9 cell lysate and protein elution buffer and positive control including purified BFDV from an infected bird was performed in parallel with the purified recombinant BFDV ORFC1 and VLPs.

2.2.26 Electron microscopy

To confirm the formation of VLP the caesium chloride purified BFDV ORFC1 VLPs were coated onto copper 400-mesh Formvar carbon coated grids by floating the grids on droplets of the sample for 10 s. Excess liquid was absorbed by Whatman filter paper and the sample stained with phospho-tunsic acid (PTA) by floating the grids on droplets of PTA for 10 s, the excess liquid was removed and the grid allowed to dry for at least 2 hr prior to viewing by EM.

2.2.27 Production of Monoclonal Antibodies against BFDV VLPs

Four BALB/c mice were injected with 50 µg of protein mixed with Freund's Incomplete adjuvant intraperitoneally. Mice were injected twice at four-weekly intervals, then serum collected four weeks after the second injection. The sera of the vaccinated mice were screened by HI, ELISA and western blot as described above. For western blotting, sera were diluted 1:100 in blocking buffer. After screening the sera, one mouse was selected and boosted by injection with a further 50 µg of protein and its spleen harvested 2 weeks later.

2.2.28 Production and screening of hybridomas

Sp2/0 mouse myeloma cells were grown and maintained in DMEM (Gibco) supplemented with 1% FCS and antibiotics (penicillin and streptomycin), at 37ºC in a 5% CO2-in-air environment.

The spleen from mouse BB01 was harvested aseptically, the pulp passed through a fine mesh filter and the cells collected into DMEM. The cell suspension and Sp2/0 myeloma cells were then pelleted separately by centrifugation. Fusion of the spleen cells and myeloma cells was accomplished by the addition of polyethylene glycol

(PEG, MW 1500kDa) with gentle mixing. Two equal volumes of the cell suspension, made up to a final volume of 40 mL with DMEM, were prepared and a 200 µL aliquot of the cell suspension added to all wells of four 96-well culture plates. The plates were incubated for 3 days, after which time the culture media was changed to Hybridoma-SFM (Gibco), supplemented with 1% FCS, antibiotics and IL-6 (composition not supplied). Media changes were performed every 2-3 days for 14 days, after which time the hybridoma supernatants were screened by ELISA.

ELISA-positive hybridomas were expanded to 1 mL, allowed to grow overnight, and re-screened by ELISA. To determine the class of antibodies, a second ELISA was performed, using a BioRad Typer isotyping kit as per the manufacturer's instructions. Any hybridomas positive for IgM were excluded from the study.

IgG-secreting hybridomas were then screened thrice by haemagglutination-inhibition assay and once by western blot. The first haemagglutination-inhibition assay was performed as previously described; the second and third assays were similar except that the second time the samples were only adsorbed with galah erythrocytes and the third time the samples were not adsorbed with either kaolin or galah erythrocytes. Western blotting was carried out as described above, with the hybridoma supernatants diluted 1:2 in TBS and serum from mouse BB01 diluted 1:100 in TBST used as the positive control. One hybridoma, which had a high ELISA titre and detected only the recombinant protein by western blot was selected and cloned by limiting dilution. After 14 days the supernatants were screened by ELISA as described above.

Clones that were positive by ELISA were expanded in 6 well culture plates and re-screened by ELISA and western blot. Western blotting was performed with supernatants tested against both the recombinant BFDV capsid protein and a polyhistidine-tagged recombinant baculovirus-expressed PCV2 capsid protein.

2.2.29 Production and purification of monoclonal antibodies

The selected hybridoma was expanded to a total volume of one litre. Before purification of the monoclonal antibody, hybridoma cells were pelleted by centrifugation at 1500rpm for 5 minutes. The supernatant was collected and passed over protein A-coated sephadex beads (BioRad) in a BioRad HPLC pump at 4ºC. The bound antibodies were washed once with excess PBS and then eluted with 50mM glycine buffer (pH 2.7), into Tris buffer (1M Tris-HCl, pH 8). Purified antibodies were then dialysed in PBS overnight at 4ºC.

2.2.30 Optimisation of the indirect enzyme-linked immunosorbent assay

The recombinant protein was diluted to 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 2.5µg/mL in 0.05M carbonate/bicarbonate buffer, applied to duplicate rows of two Microlon 600 ELISA plates (Greiner BioOne) and allowed to coat at 4ºC overnight. The following morning, the plate was washed with wash buffer (PBS, 0.05% (v/v) Tween 20), then blocking buffer (PBS, 0.05% (v/v) Tween 20, 5% (w/v) skim milk powder) added to all wells and the plate incubated for 1 hour at room temperature. Two solutions of the monoclonal antibody were prepared; a 1:25 and a 1:30 dilution in blocking buffer. After washing the protein coated plates again, 50 µL of blocking buffer was added to all wells, a 1:25 dilution of the monoclonal antibody was added to the first well of the first row of each protein dilution and a 1:30 dilution added to the

first well of the second row. The mixtures from the first well of each row were then serially diluted across the plates and the plates incubated for 1 hour at room temperature. After washing again, HRP-conjugated goat anti-mouse IgG (Sigma), at the manufacturer's recommended dilution in blocking buffer, was added to all wells of both plates and the plates incubated for 1 hour at room temperature. The plates were washed again, then 50 µL/well of a solution containing ABTS (BioRad) was added and colour allowed to develop for 15 minutes at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405nm measured using a spectrophotometer.

2.2.31 Optimisation of western immunoblotting

A nitrocellulose membrane containing the recombinant protein was prepared and western blotting was carried out as described above. The monoclonal antibody was diluted 1:50 and 1:62.5 in TBS, then serial 1:2 dilutions were made of each solution. One hundred and fifty microlitres of each consecutive dilution were added to individual lanes of a Bio-Rad Mini-PROTEAN II multiscreen apparatus and incubated at room temperature for 1 hour. Colour development was carried out as described previously.

2.2.32 Optimisation of indirect immunohistochemistry

Three different dilutions of the monoclonal antibody (1:50, 1:200 and 1:500) were used in an indirect immunohistochemistry (IHC) procedure to optimise the amount of antibody. Five-micron sections of formalin-fixed and paraffin embedded liver tissue, from a rainbow lorikeet (Trichoglossus haematodus) known to be infected with BFDV, were cut using a microtome (Leica RM 2135), 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. Seven tissue sections were prepared; one negative control (no monoclonal antibody), then duplicate sections for each dilution tested. Of these duplicate sections, one of each was treated for antigen retrieval by microwaving three times for three minutes each time in citrate buffer pH 9. Endogenous peroxidase activity was quenched by the addition of 0.3% (v/v) hydrogen peroxide for 5 minutes, then the sections washed with deionised water. Each of the dilutions of monoclonal antibody was then applied to the duplicate sections for 10 minutes at room temperature, with TBS applied to the negative control section. The sections were then washed twice with TBS and tapped dry. EnVision anti-mouse HRP (Dako) was then added and the sections incubated at room temperature for 30 minutes, after which the sections were washed twice with TBS. DAB solution (Dako) was added and the sections incubated at room temperature for 3 minutes, then rinsed in deionised water. The sections were then dehydrated in increasing concentrations of ethanol, counterstained with haematoxylin and mounted.

2.3 Results

2.3.1 Expression of BFDV capsid protein by baculovirus expression system

The BFDV cap protein was successfully expressed and purified using a eukaryotic baculovirus expression system. Optimal conditions for the expression of BFDV cap involved infecting a Sf9 cell culture in mid-logarithmic phase with the recombinant baculovirus at an

MOI of 5 and allowing the culture to incubate at 28°C for 72 h after the Sf9 cells had ceased dividing.

The expressed and purified BFDV Cap was examined by mass spectrometry (MALDI-TOF). The protein was digested with trypsin and the spectra obtained from this digest were compared to a theoretical trypsin digest of BFDV cap proteins based on sequences located in GenBank. The recombinant BFDV Cap produced in the baculovirus system had a high correlation (>65%) to submitted sequences of putative BFDV capsid proteins. Figures 5.5 (Coomassie blue stained) and 5.6 (Western immunoblot utilising a monoclonal anti-His antibody to detect the His-tagged recombinant BFDV cap) show the various purification steps involved in isolating the recombinant protein with a nickel resin. A number of wash steps were required to remove as much of the contaminating cellular proteins as possible, and the final 2 wash steps (Figures 4.3. and 4.4, lanes 8 and 9) contained a low concentration of imidazole (50 mM) assisted in eluting any His-rich proteins from the nickel resin. The recombinant protein was eluted from the resin using a buffer containing imidazole at a final concentration of 250 mM (Lanes 11, 12). The concentration of imidazole was determined empirically after examining the effects of 0.1-1 M imidazole on elution of the protein. As can be seen from Figures 4.3 and 4.4, protein was not eluted immediately in the first 0.5 mL fraction but was found subsequently in the second and third 0.5 mL fractions.

The concentration of the final purified recombinant protein was estimated at 41 μg/mL. The HA titre of the protein was 1:10,240 HAU/50 μL.

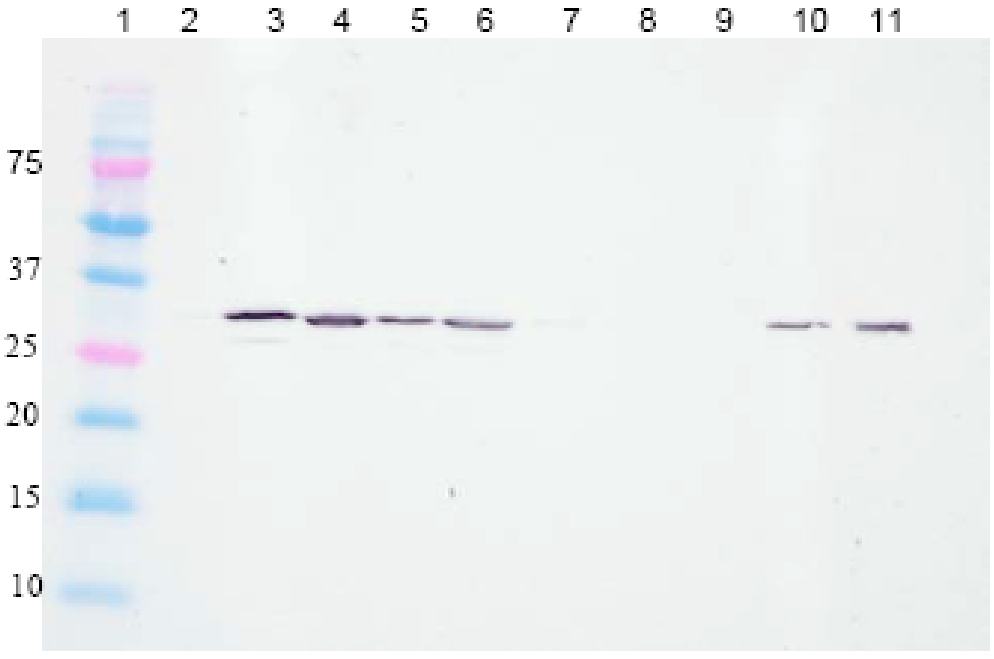

Figure 6. Western immunoblot using anti-His Ab. 1. MW 2. uninfected SF9 cell 3. recBaculovirus infected cells 4. unbound fraction 5-8 wash steps 9-10 eluted protein fractions. 11 eluted protein.

2.3.2 Detection of antibodies against recombinant BFDV Capsid protein

BFDV ORFC1 was the presumptive capsid protein of the virus based on sequence homology to other circoviruses (Bassami *et al.,* 1998) and a truncated version of ORFC1 has been demonstrated to be antigenic (Johne *et al.,* 2004). Thirteen psittacine sera had been previously been used in HAI assay (11 HI postive and 2 HI negative) were tested for reactivity with the recombinant BFDV ORFC1 protein. The HI postive antisera specifically recognised the 32 kDa recombinant protein. The purified His-tagged BFDV Capsid reacted specifically in western immuno-blot analysis with polyclonal chicken anti-BFDV.

All 4 chickens were HI antibody negative 7 days prior to and at the time of inoculation. Although vaccinated twice at about 14 day intervals, 2 of the 4 birds did not develop a detectable HI antibody titre within 14 days of the second vaccination when tested against both native virus HA antigen and the recombinant baculovirus-expressed protein antigen. One chicken (C210) developed a HI antibody titre of 80 HIU/50 µL 14 days after the second inoculation against the native virus HA antigen and the recombinant baculovirus-expressed protein antigen. A second chicken (F312) also developed a HI antibody titre of 80 and 320 HIU/50 µL 14 days after the first and second inoculation respectively when tested with the native virus HA antigen and a titre of 40 HIU/50 µL 14 days after the second injection of antigen when tested against the recombinant baculovirus-expressed protein antigen.

2.3.3 Recombinant BFDV ORF C1 assembled into virus like particles

To demonstrate that the recombinant BFDV capsid protein self-assemble to form VLP the Sf9 cell lysate containing the recombinant protein was purified by a sucrose cushion and CsCl gradient. The migration in the CsCl gradient was slightly higher than the BFDV virus. The fraction was analysed by EM and SDS-Page (data not shown) and the proteins were predominately found between 1.215 and 1.325 g/ml whereas wild type BFDV fractionates between 1.35-1.37 g/cc (Raidal and Cross, 1994). To analyse the formation of VLPs and examine the structural integrity the fractions were coated onto 400-mesh formvar coated carbon grids for EM. Negative staining demonstrated that the capsid proteins did self-assemble to produce VLPs. The VLPs were also visible in the fractions purified with the NiNTA resin (data not shown). The images showed that the structures were spherical virus like particles measuring 18-20 nm and similar to wild type BFDV with no partially assembled particles present. These results provide evidence that BFDV ORFC1 encode the capsid structural protein of the virus and indicate that the putative Rep protein is not involved in the assembly of the virus.

2.3.4 Recombinant BFDV ORF C1 and VLPS has haemagglutination activity

Recombinant BFDV ORFC1 purified by either the NiNTA column method or by CsCl purification demonstrated haemagglutination activity when used as the antigen in HA assay (Fig 4). The protein had been quantified by a Bradford assay and serially diluted (2-fold, 3-fold, 5-fold, 10-fold and 100-fold) to determine the minimal amount of VLPs to cause haemagglutination. Haemagglutination was detected at concentrations between 15.2 to 21.2 ng but not below dilutions containing 14.0 ng or less. These results indicate that the determinants for HA are in the Capsid protein and not in the putative Rep protein of BFDV.

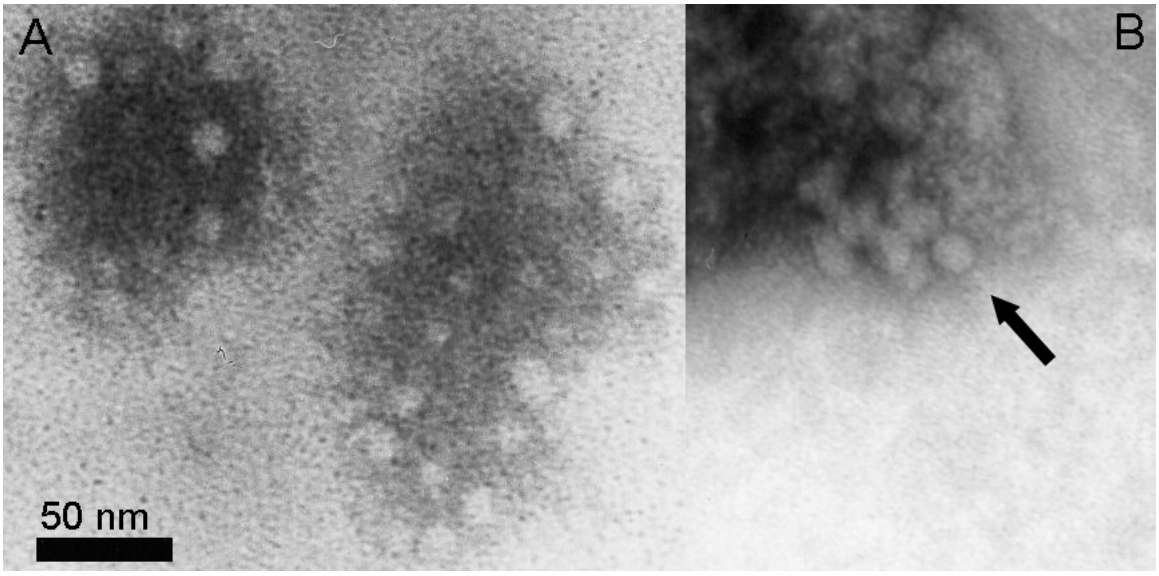

Figure 7. Negatively stained VLP capsid protein demonstrating partially (A) and fully assembled particles (B).

2.3.5 BFDV VLPs can be successfully used in HI assay

BFDV has never been successfully cultured in vitro thus all HI assay require the purification of the virus from a persistently infected bird. Based on the HA results, 100 ng of the BFDV VLPs were added to each well which was equivalent to 2 HA units. A series of HA positive and negative series were used in HAI assay with the VLPs. The haemagglutination activity of the BFDV VLP was successfully neutralised by antibodies in the sera from birds that had already demonstrated HAI activity with wild type BFDV (Fig 5). Comparison of serum HI titres in a variety of serum and plasma samples known to contained HI antibody against either wild type or recombinant VLP as HI antigen demonstrated no differences in observed titre between the two sources of antigen.

2.3.6 Monoclonal Antibody to a Recombinant BFDV Capsid Protein

The spleen cells from mouse BB1 were fused with Sp2/0 myeloma cells and many hybridomas grew very well. ELISA showed that most (>70%) hybridomas were producing antibody that recognised the recombinant protein (data not shown), so the 29 hybridomas with the strongest ELISA result were selected, then expanded and re-screened and their antibody isotype determined. All 29 hybridomas were still producing antibodies after expansion and isotyping showed that 28 of the 29 hybridomas produced IgG. These 28 hybridomas were screened 3 times by HI assay as described above. No HI activity was detectable in any of the supernatants, regardless of whether or not they were treated prior to testing. When samples were not adsorbed with kaolin (ie haemadsorption only or no treatment), non-specific haemaggluination (HA) was present in the samples. Haemadsorption prior to testing reduced this non-specific HA activity by a factor of log2.

Western blotting of hybridoma supernatants showed that most hybridomas were secreting a polyclonal array of antibodies and only some of these successfully detected the recombinant protein (Figure 1A). Hybridoma 3F8 was selected as it

reacted strongly in both the ELISA and western blot and it appeared that almost all of the antibody it produced was specific to the recombinant protein.

Hybridoma 3F8 was cloned by limiting dilution and screened by ELISA and western blot. Three clones reacted positively by ELISA and all clones detected only the recombinant protein and not the PCV2 capsid protein or polyhistidine tag by western blot (Figure 1B). Clone 3F8-1 had the strongest reaction by ELISA and western blot and was chosen for expansion and production of monoclonal antibodies.

Hybridoma 3F8-1, when untreated by either kaolin or haemadsorption, had an HI titre of between log22-log24, depending on the amount of virus used in the test.

2.3.7 Indirect enzyme-linked immunosorbent assay

The optimal amount of protein used in the indirect ELISA was determined to be 250ng/well and the optimal dilution of monoclonal antibody was 1:400. The indirect ELISA performed well at many other combinations of protein and monoclonal antibody dilution, but the above protein amount and antibody dilution were selected as they had a good positive absorbance value and a useful dynamic range.

2.3.8 Western immunoblotting and indirect immunohistochemistry

The optimal dilution of monoclonal antibody for use in both western immunoblotting and indirect IHC was determined to be 1:500. When used in western immunoblotting, the monoclonal antibody detected the recombinant protein at all dilutions up to 1:4000. The monoclonal antibody detected BFDV antigen in formalin fixed, paraffin embedded tissues at all dilutions tested. Background staining and edge effect were significant when the monoclonal antibody was used at 1:50 and 1:200 and made evaluation of the results more difficult. Antigen retrieval decreased the endogenous peroxidase activity of erythrocytes, but increased non-specific background staining and decreased the amount of specific positive staining at all dilutions tested.

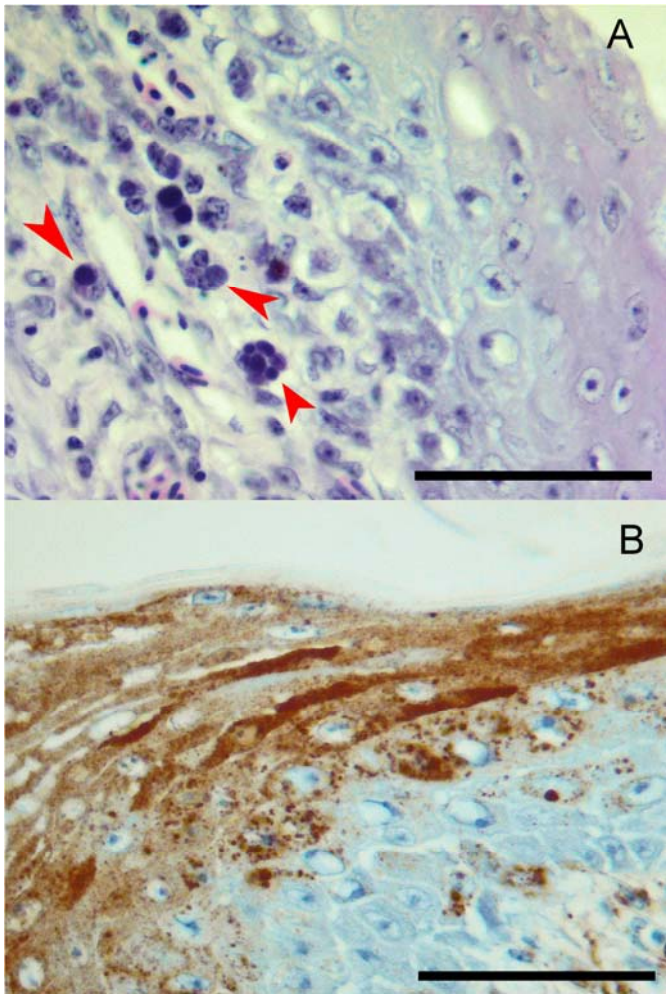

Figure 8. Histological section of feather from a psittacine bird with PBFD stained with Haematoxylin and Eosin (A) Arrows indicate BFDV inclusions. B) Brown stain highlights BFDV antigen by immunohistochemistry using anti-BFDV monoclonal antibodies.

2.4 Discussion

Given the high rates of BFFV infection in psittacine birds worldwide, sensitive and specific detection methods which are readily standardised are highly desirable. Since the BFDV capsid protein is the major antigenic determinant of the virus (Stewart *et al.*, 2007), the capsid protein and a specific antibody to it are the two most important components of any diagnostic assay for BFDV. Recombinant BFDV capsid proteins (Heath *et al.*, 2006, Johne *et al.*, 2004, Stewart *et al.*, 2007) can be produced in large quantities and quantified reasonably accurately and may be substituted for whole BFDV virus, which cannot be easily produced. The monoclonal antibody to the BFDV capsid protein described is also able to be produced in the laboratory in large quantities and accurately quantified. This antibody, in combination with the recombinant BFDV capsid protein, should enable the further development of standardised diagnostic tests.

There has been only one other report on the development of monoclonal antibodies to BFDV (Ritchie *et al.*, 1992). This monoclonal antibody was developed using whole virus prepared from the feathers of infected birds and was optimised for use in an indirect ELISA and

immunohistochemistry. Unfortunately, there have been no subsequent reports on the use of this monoclonal antibody in other applications. Ritchie *et al*. (1992) reported that the monoclonal antibody they developed was not able to inhibit haemagglutination by BFDV. However, this study demonstrates that this may simply have been due to the amount of monoclonal antibody used in the HI test, as the supernatant of hybridoma 3F8-1 also did not inhibit haemagglutination until it was purified and concentrated. The monoclonal antibody described in this study will no doubt be suitable for ongoing use in the western blotting and immunohistochemistry as described above and will enable standardisation of these applications. The antibody detected virus from a cockatoo and a lorikeet (this study) and a cockatiel (Shearer *et al.,* 2007), but ongoing testing using virus from as many species of psittacine birds as possible is still necessary. Cross-reactivity work using sera from rainbow lorikeets, short-billed corellas (*Cacatua sanguinea*), a sulphur-crested cockatoo, a red lory (*Eos bornea*) and a galah-corella hybrid and virus from rainbow lorikeets, a red lory, two swift parrots (*Lathamus discolor*), a sulphur-crested cockatoo and a scarlet-chested parrot (*Neophema splendida*) failed to find evidence of antigenic serotypes (Khalesi *et al.,* 2005). However, a similar experiment using sera from the above birds and virus eluted from the feather of a cockatiel found that sera from some of the birds did not inhibit agglutination by the cockatiel isolate, suggesting that the cockatiel isolate may be sufficiently different antigenically to be considered a separate serotype (Shearer *et al.,* 2007). Given, though, that the antibody detects virus from cockatiels, it should have widespread application for the detection of most, if not all other BFDV isolates.

Interestingly, the optimal dilution of the antibody is the same when used in both western blotting and immunohistochemistry. Often the formalin-induced cross-links within and between proteins require the use of antigen retrieval techniques (Rait *et al.,* 2004) and/or a more concentrated primary antibody solution. According to Sompuram *et al*. (2006), the epitope detected by the monoclonal antibody described in this study is most likely linear, with a high concentration of proline, tyrosine, glutamine and/or leucine.

The monoclonal antibody should have widespread application in both diagnostic and research work. Its use in western immunoblotting and HI assays will likely be limited to research, since western blotting is not practical for the routine detection of viral antigen and large amounts of the antibody are needed to inhibit haemagglutination. The initial results of IHC testing are promising and the antibody should have widespread diagnostic application after its efficacy has been tested with a suitable range of virus isolates. Future work will examine other applications of the antibody, including the development of ELISAs for the detection of anti-BFDV antibodies in psittacine sera and the presence of excreted virus in feather and faecal samples.

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Chapter 3. A blocking ELISA for the detection of antibodies to psittacine beak and feather disease virus (BFDV)

3.1 INTRODUCTION

Currently, an haemagglutination-inhibition assay (HI) (Raidal *et al.,* 1993b, Ritchie *et al.,* 1991) is the only method available for the detection of anti-BFDV antibodies in psittacine sera. This assay detects both IgM and IgY antibodies from a wide range of species of psittacine birds, but it suffers from an appreciable amount of inter-test variation due to the variability in quality and quantity between virus preparations and the sensitivity of the erythrocytes used in the test to the virus. To overcome these limitations, we describe a novel blocking (or competitive) ELISA which was developed utilising a baculovirus-expressed recombinant BFDV capsid protein (Stewart *et al.,* 2007) and a newly developed monoclonal antibody raised against this protein (Shearer *et al.,* 2007).

3.2 MATERIALS & METHODS

Insofar as was practical, the bELISA was developed and validated in accordance with guidelines established by the Office International des Epizooites (O.I.E.) (Jacobson, 2004).

3.2.1 Samples

Submissions to our laboratory of blood, collected on filter paper and air-dried and serum or plasma from 5 species of psittacine birds (including sulphur-crested cockatoos (*Cacatua galerita*), galahs (*Eolophus roseicapillus*) rainbow lorikeets (*Trichoglossus haematodus*), eclectus parrots (*Eclectus roratus*) and gang-gang cockatoos (*Callocephalon fimbriatum*) known to have naturally occurring HI anti-BFDV antibodies were used to optimise the blocking ELISA (bELISA). These were selected to include a range of HI antibody titres. A sample of serum from a chicken inoculated with a baculovirus-expressed recombinant BFDV capsid protein (Stewart *et al.,* 2007) was used as a low-HI antibody sample and normal chicken serum was included as a known negative control.

To validate the bELISA, 166 samples from eastern long-billed corellas (*Cacatua tenuiostris*) challenged with live virus were tested using the optimised bELISA. In addition, 82 samples from cockatiels known to be HI negative (Shearer *et al.,* 2008) were included to evaluate the diagnostic specificity of the test. Samples included both dried blood on filter paper and plasma.

For blood collected onto filter paper, two spots of blood on filter paper were excised using either scissors or a hole punch and placed into a microcentrifuge tube (Eppendorf). One hundred microlitres of ELISA blocking buffer containing PBS (0.05% v/v), Tween 20 (5% w/v), skim milk powder (Diploma) was then added in order to make a 1:5 (w/v) suspension

and serum eluted from the paper by incubating the mixture for 1 hour at room temperature. Serum or plasma samples were diluted 1:5 in blocking buffer.

3.2.2 Production of recombinant BFDV capsid protein

A full-length, baculovirus-expressed BFDV capsid protein was expressed and purified as described by Stewart *et al* (2007).

3.2.3 Optimisation of the Indirect Enzyme-Linked Immunosorbent Assay

The indirect ELISA was optimised as described by Shearer *et al* (2007). The recombinant protein was diluted to 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 2.5 µg/ml in 0.05M carbonate/bicarbonate buffer, applied to duplicate rows of two Microlon 600 ELISA plates (Greiner BioOne) and allowed to coat at 4ºC overnight. The plate was then washed with wash buffer (PBS, 0.05% (v/v) Tween 20), blocking buffer added to all wells and the plate incubated for 1 hour at room temperature. Two solutions of the monoclonal antibody were prepared; a 1:25 and a 1:30 dilution in blocking buffer. After washing the protein coated plates again, 50ul of blocking buffer was added to all wells, a 1:25 dilution of the monoclonal antibody was added to the first well of the first row of each protein dilution and a 1:30 dilution added to the first well of the second row. The mixtures from the first well of each row were then serially diluted across the plates and the plates incubated for 1 hour at room temperature. After washing again, HRP-conjugated goat anti-mouse IgG (Sigma), at the manufacturer's recommended dilution in blocking buffer, was added to all wells of both plates and the plates incubated for 1 hour at room temperature. The plates were washed again, then 50 µL/well of a solution containing ABTS (BioRad) was added and colour allowed to develop for 15 minutes at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405nm measured using a spectrophotometer.

3.2.4 Optimisation of the Blocking Enzyme-Linked Immunosorbent Assay for the Detection of Specific Antibodies

Recombinant baculovirus-expressed BFDV capsid protein was diluted to 2.5 µg/mL in 0.05 M carbonate/bicarbonate buffer, added to all wells of a Microlon 600 ELISA plate (Greiner BioOne) and allowed to coat at 4ºC overnight. The plate was then washed with wash buffer, blocking buffer added to all wells and the plate incubated for 1 hour at room temperature. After another wash step, 50 µL of blocking buffer was added to all wells of the ELISA plate and 50 µL sera, eluted as above, was added to the first well of each row and serially diluted across the plate. The plate was then incubated for 1 hour at room temperature. After removing the sera, the plate was washed again and 50 µL/well of a monoclonal antibody against the recombinant BFDV capsid protein (Shearer *et al.,* 2007), diluted 1:400 in blocking buffer, was added and the plate incubated for 1 hour at room temperature. After washing, 50 µL of polyclonal anti-mouse IgG (Sigma), at the manufacturer's recommended dilution in blocking buffer, was added to each well and the plate incubated for 1 hour at room temperature. After washing, 50 µL/well of a solution containing ABTS (BioRad) was added and colour allowed to develop for 15 minutes at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405nm measured using a spectrophotometer. The percentage inhibition (PI) of the test sera samples and negative cut-off value (based on the PI of the known negative samples) were then calculated.

3.2.5 Validation of the bELISA

To validate the bELISA, 166 samples from eastern long-billed corellas (*Cacatua tenuiostris*) challenged with live virus were tested using the optimised bELISA. Samples included both dried blood on filter paper and plasma. After testing, the PI of the samples were compared to the corresponding HI titres. In addition, 82 samples from cockatiels that had been assessed as negative by HI (Shearer *et al.,* 2008).

For blood collected onto filter paper one spot of blood on filter paper was cut out using scissors or hole punch and collected into a microcentrifuge tube (Eppendorf). To this 100 µL of ELISA blocking buffer was then added (to make a 1:10 (w/v) suspension) and serum eluted from the paper by incubating the mixture for 1 hour at room temperature. Serum or plasma samples were diluted 1:10 in blocking buffer.

Tests to evaluate the precision, repeatability and accuracy of the assay were also conducted; however it was not practical to evaluate reproducibility between laboratories. Repeatability assays were conducted using pooled sera from 10 vaccinated birds. The intra-assay coefficient of variation was determined by performing 10 replicates of pooled sera and the inter-assay coefficient of variation was determined by repeating this group of replicates 10 times. Data from the assay of intra-assay repeatability was plotted to determine the assay's precision. The linearity of the assay was also investigated; 10 replicates of the pooled sera were serially diluted 1:2 and the PI values plotted against the log2 of the dilution factor.

3.3 Results

3.3.1 Indirect Enzyme-Linked Immunosorbent Assay

The optimal amount of protein used in the indirect ELISA was determined to be 250ng/well and the optimal dilution of monoclonal antibody was 1:400. The indirect ELISA performed well at many other combinations of protein and monoclonal antibody dilution, but the above protein amount and antibody dilution were selected as they had a good positive absorbance value and a useful dynamic range.

3.3.2 A Novel Blocking ELISA for the Detection of Antibodies to BFDV

The optimal dilution of serum for use in the bELISA was determined to be 1:10. This gave a useful dynamic range and a lower limit of detection (greater analytical sensitivity) than the HI test.

All cockatiels that tested negative by HI were also negative by bELISA. The corellas vaccinated with the recombinant protein had peak mean PI levels of between 6.01% and 44.26% at challenge (after the second vaccination) and between 62.43% and 90.22% after challenge with live virus. Control birds had peak antibody levels of between 55.25% and 83.26% after challenge with live virus. Importantly, although the samples from vaccinated birds were HI-negative at the time of the second vaccination, the bELISA detected low levels of antibodies in these samples. Antibody levels measured by bELISA correlated strongly with HI titres ($r2 = 0.8156$, $p<0.05$). Based on the results of the vaccine trial samples only,

sensitivity was 99.09% and specificity was 71.43% when compared to the HI assay, with a positive and negative predictive value of 87.2% and 97.56% respectively. Using the results of the cockatiel sero-survey, the bELISA was 100% specific and combining these results with the results of the vaccine trial gave the same sensitivity and increased the specificity to 88.41% and the negative predictive value to 99.19%. The mean PI value of the pooled sera was 64.28% and almost all repeated samples fell within ± 2 standard deviations of the mean value. The intra- and inter-assay coefficients of variation were 3.61% and 6.94%, respectively and the bELISA was determined to be linear within the working range of the samples tested.

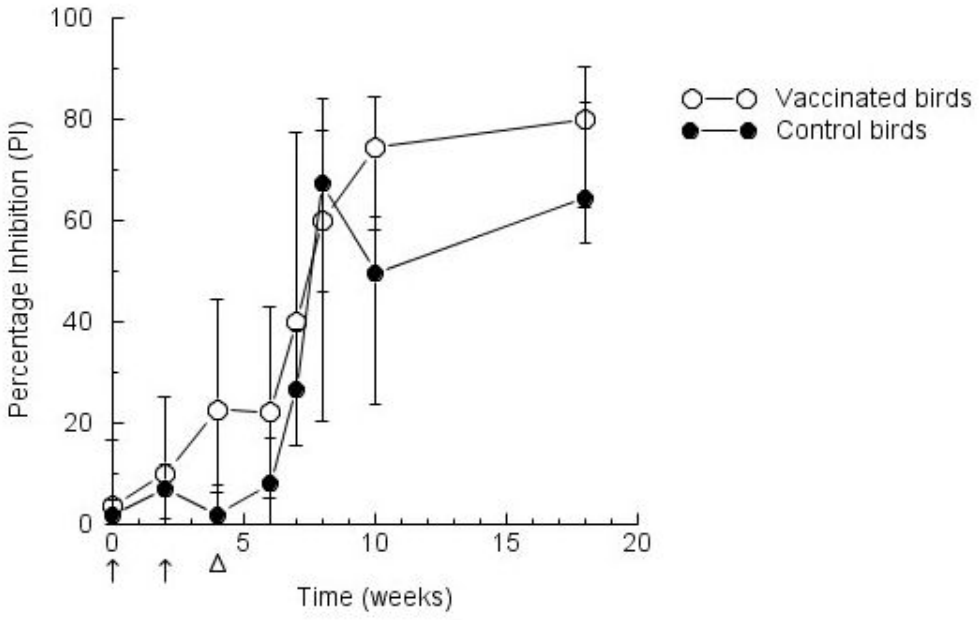

Figure 9. Blocking ELISA (bELISA) mean percentage inhibition values of vaccinated and control birds after vaccination and challenge. Vaccinations are indicated by an arrow (↑) and challenge with a triangle (Δ). Bars indicate standard error.

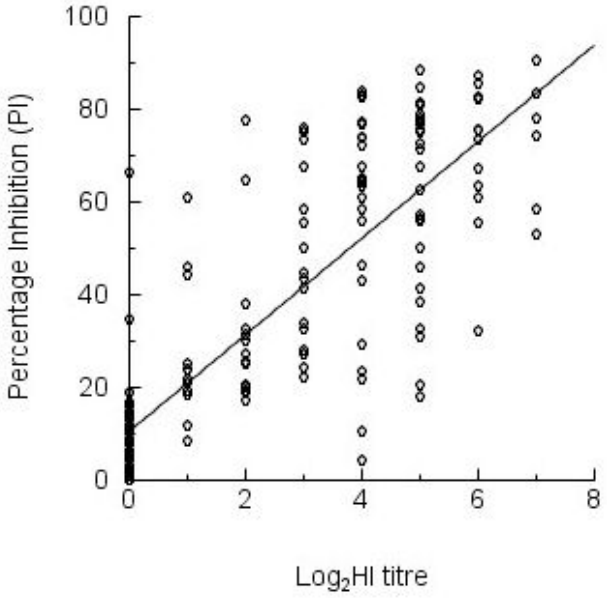

Figure 10. Correlation of haemagglutination inhibition (HI titres) with blocking ELISA (bELISA) percentage inhibition (PI)

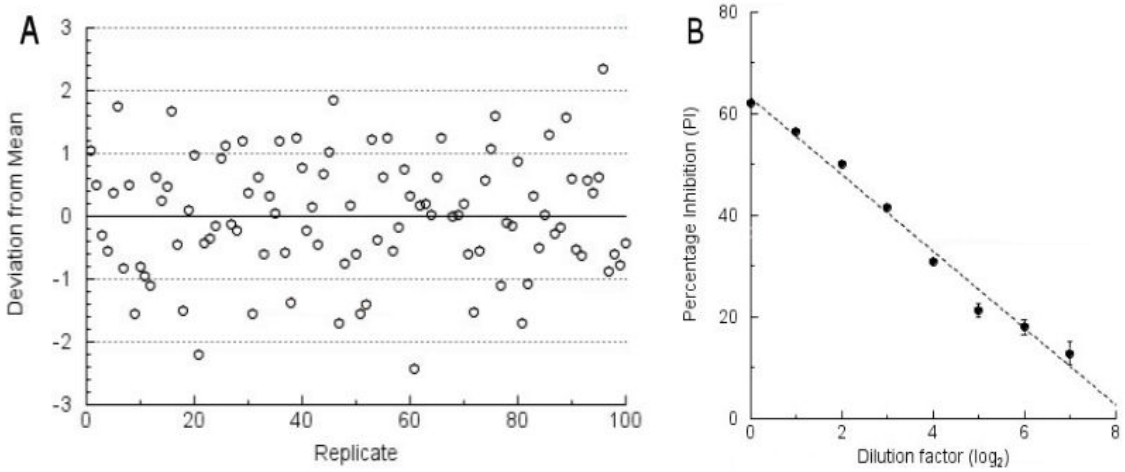

Figure 11. A: Precision assay, note that almost all values fall within ±2 standard deviations from the mean; B: Linearity assay using sera pooled from 10 vaccinated birds. The percentage inhibition (PI) values are linear within the dynamic range of the test.

3.4 DISCUSSION

Presented here are the methods used to develop a novel blocking ELISA for the detection of anti-BFDV antibodies in psittacine sera. Blocking ELISAs have been developed for the

detection of antibodies to other avian viruses, such as avian metapneumovirus Type-C (Turpin *et al.,* 2003), avian polyomavirus (Khan *et al.,* 2000), chicken anaemia virus (Tannock *et al.,* 2003, Todd *et al.,* 1999) and turkey coronavirus (Guy *et al.,* 2002). These blocking ELISAs have the distinct advantage over indirect ELISAs, in that secondary antibodies specific to the immunoglobulins of the species being tested are not required. For current large-scale screening and sero-surveillance, blocking ELISAs have largely replaced indirect ELISAs (Gorham, 2004)

The blocking ELISA developed here is both sensitive and specific and should prove to be a useful diagnostic test for BFDV. The monoclonal antibody used in the assay has previously been shown to have a high analytical specificity (Shearer *et al.,* 2007). The analytical sensitivity was greater than the HI test, as it detected antibodies in vaccinated birds that were HI negative, however because of this, the analytical sensitivity could not be determined precisely. The comparative diagnostic sensitivity and specificity was good, however because of the greater analytical sensitivity of the bELISA, the absolute diagnostic sensitivity and specificity is likely to be higher. Given the calculated diagnostic sensitivity, 166 samples is adequate to validate the assay with 95% confidence of an accurate result (Jacobson, 1998). However, assuming the highest calculated diagnostic specificity of 88%, the number of known negative animals required is 1 014. The actual number of known negative animals required is likely to be much less, since the actual diagnostic specificity is likely to be higher. Since it is so difficult to find psittacine birds that have not been exposed to the virus, ongoing testing with birds of known antibody status is desirable. The precision, repeatability and accuracy of the assay was very good, but reproducibility could not be assessed since testing in other laboratories was impractical. The cut-off value was determined from a 95% confidence interval of the negative control wells (Coligan *et al.,* 2001) since it was almost impossible to find a group of birds known to be truly seronegative. Assuming, though, that the cockatiels were truly seronegative, the cut-off value calculated from a 95% confidence interval of the mean PI of those 82 samples was similar to that calculated using the negative control wells only. As more samples are tested using this assay over time, these methods of calculating a cut-off value can be compared with receiver-operator curves to see which method is more appropriate. The positive and negative predictive values were good and given the high seroprevalence of BFDV, should translate well to samples from naturally infected birds.

The assay correlated strongly with HI titres from a group of corellas vaccinated with a recombinant BFDV capsid protein and challenged with live virus. The fact that the bELISA correlated strongly with the HI assay is somewhat surprising given the differing nature of the two tests. The HI assay gives a defined cut-off, with the difference between the last positive well and the first negative well being a 1:2 dilution of the sample. The bELISA, on the other hand, would allow more precise characterisation of antibody levels, as it allows for continuous readings of PI values for samples with antibody levels which fall in between HI end-points. The intra- and inter-assay coefficients of variation were good and in line with OIE assay validation guidelines (Jacobson, 2004). Absorbance values fell within the linear range of the assay for the samples tested, however evaluation of the assay's behaviour for samples with PI values greater than 64.28% was not possible.

Only one other ELISA has been developed to test for the presence of anti-BFDV antibodies in psittacine sera (Johne *et al.,* 2004). This was an indirect ELISA, utilising a truncated recombinant BFDV capsid protein and a secondary antibody directed against psittacine IgY.

This ELISA tested 11 serum samples from 7 different psittacine species and thus has yet to be validated with a large number of samples of birds with known antibody status. Additionally, no studies have yet been conducted investigating the cross-reactivity of psittacine IgY and given that there are 78 genera and 332 species within the Psittacidae, such studies may be prohibitively difficult. This means, though, that a sample from a rare species of psittacine bird which tested negative by indirect ELISA could not be guaranteed to be truly negative. Consequently, we believe that a blocking ELISA, as described here, is likely to be a more reliable diagnostic test. The blocking ELISA also has the advantage that serum containing both IgM and IgY can be reliably tested using the one assay, as it does not rely on secondary antibodies directed against either class of immunoglobulin.

Although the ELISA described here has been validated with 166 samples (251 including cockatiels), further testing is necessary to ensure that the assay performs well with sera from as many psittacine species as possible. Any potential problems with consistency of the test between species would theoretically be the result of differing cross-reactivities between the test serum and the recombinant protein. Stewart *et al*. (2007) failed to find any differences in cross reactivity between the recombinant protein and a number of psittacine anti-BFDV antisera in both western blotting and HI. Additionally, cross-reactivity work using sera from rainbow lorikeets, short-billed corellas, a sulphur-crested cockatoo, a red lory (*Eos bornea*) and a galah-corella hybrid and virus from rainbow lorikeets, a red lory, two swift parrots (*Lathamus discolor*), a sulphur-crested cockatoo and a scarlet-chested parrot (*Neophema splendida*) failed to find evidence of antigenic serotypes (Khalesi *et al.,* 2005). However, a similar experiment using sera from the above birds and virus eluted from the feather of a cockatiel found that sera from some of the birds did not inhibit agglutination by the cockatiel isolate, suggesting that the cockatiel isolate may be sufficiently different antigenically to be considered a separate serotype (Shearer *et al.,* 2008). The fact that a monoclonal antibody developed by the same authors recognised BFDV from a sulphur-crested cockatoo, rainbow lorikeet and a cockatiel (Shearer *et al.,* 2008) indicates that there is some antigenic homology between isolates. A sero-survey of 88 cockatiels by the same authors also failed to find any birds with detectable HI titres and these results are confirmed by the results of the bELISA testing conducted in this study. Answers to questions about the existence of a cockatiel-adapted BFDV serotype and the relative infectivity and antigenic characteristics of various virus isolates remain to be discovered. These answers may only be found by conducting HI assays using sera from many cockatiels, tested against virus eluted from the feathers from a range of psittacine birds. However, finding cockatiel anti-BFDV serum may be difficult to achieve given the fact that the cage mate of a known BFDV-positive bird tested negative by HI using virus prepared from both cockatoo and cockatiel feathers and also tested negative by bELISA (Shearer *et al.,* 2008; this study).

The bELISA has a number of advantages over the HI test. First and most importantly, the bELISA is much more easily standardised as the amounts of both the recombinant protein and monoclonal antibody used in the assay can be quantified reasonably accurately. Even though both the recombinant protein and monoclonal antibody are initially expensive to develop and produce, they are a much more reliable and consistent source of reagents than the virus preparation, polyclonal antibodies and erythrocytes used in HI assays. Virus used in the HI assay must be purified from the feathers from persistently infected birds. This process is expensive, ethically questionable, time consuming, results in low yields of virus and the extraction procedure can be contaminated with host proteins. The HI assay also requires a

flock of suitable birds to be kept in order to use their erythrocytes for haemadsorption and testing. Within this flock, the sensitivity of erythrocytes to BFDV may vary between individuals within a species (Sanada and Sanada, 2000) and the sensitivity of an individual bird's erythrocytes may also vary over time. Variation in the amount of virus between preparations and decreased HA activity of the virus over time compounds this problem. Even though this variation may be standardised to an extent by titrating virus and antibody activity against each other and against the erythrocytes from multiple birds prior to testing, HI assays are still prone to an appreciable amount of inter-test variation. Another factor to consider is that the amount of antibodies present in a sample as measured by ELISA may not correlate directly with neutralisation of the virus. Studies on PCV2 have shown that variations in the length and composition of the capsid protein can cause disparities between ELISA and serum neutralisation titres (Fan *et al.,* 2008). This should not be a problem with the bELISA described here, as it uses a full-length capsid protein as the antigen and correlates well with the HI assay.

Another advantage of the bELISA over the HI assay is the amount of time taken to perform the assay. Even though there are four 1-hour incubation steps in the bELISA, sample preparation time is much reduced as samples are eluted directly into the ELISA blocking buffer. The time taken to run the bELISA could be shortened further by pre-coating and blocking the plates in bulk and storing them for future use (KPL, 2006), or by directly conjugating the monoclonal antibody to HRP. The total incubation time would therefore be reduced to 2 hours. However, the performance of the protein-coated plates after storage and the effect of the directly conjugated monoclonal antibody on peak absorbance values would need to be assessed. The bELISA could also be adapted for the performance of cross-reactivity assays; however possible differences in affinity of the monoclonal antibody for the different virus samples used as antigen in the assay would need to be determined and taken into account.

Overall, the bELISA described here should be a useful tool for the sero-diagnosis of BFDV infection. It is more readily standardised, simpler to perform, more repeatable and has a greater analytical sensitivity than the HI assay. In the future it should also provide valuable information in subsequent studies on the pathophysiology of the virus, such as the fluctuations in antibody levels at various stages of disease and the transfer of maternal antibodies and their effect on infection and immunity. In this experiment it was able to provide the valuable information that the vaccinated corellas had developed antibodies against the recombinant protein after the first vaccination and it should also prove to be very useful in the further optimisation of BFDV vaccination protocols.

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Chapter 4. A universal quantitative, real-time polymerase chain assay for beak and feather disease virus (BFDV)

4.1 INTRODUCTION

BFDV agglutinates the erythrocytes of many birds (Kondiah *et al.*, 2005, Raidal and Cross, 1994, Ritchie *et al.*, 1991, Sanada and Sanada, 2000, Sexton *et al.*, 1994, Soares *et al.*, 1998), which has allowed the development of haemagglutination (HA) and haemagglutination-inhibition (HI) assays for the detection of virus and anti-BFDV antibodies respectively (Raidal *et al.*, 1993). The ambisense viral genome contains two major coding regions: ORFV1, which encodes the replication-associated protein and ORFC1, which encodes the capsid protein. PCR assays based on the DNA sequence of both the V1 and C1 ORFs have been developed (Albertyn *et al.*, 2004, de Kloet and de Kloet, 2004, Kondiah *et al.*, 2006, Ogawa *et al.*, 2005, Ritchie *et al.*, 2003, Ypelaar *et al.*, 1999), as well as nested (Kiatipattanasakul-Banlunara *et al.*, 2002), duplex (Ogawa *et al.*, 2005) and real-time (Raue *et al.*, 2004) PCR assays. Despite the difficulties associated with performing and standardising HA assays, there are no alternative tests available for the quantitation of the virus. Quantitative, real-time PCR (qPCR) assays allow the rapid and sensitive quantitation of genetic material and are in widespread use in studies of gene expression and the detection of infectious agents (Mackay, 2004, Nolan *et al.*, 2006, Wong and Medrano, 2005). As such, a qPCR assay could be extremely useful for the detection of virus and characterisation of infection and excretion kinetics. Presented here is a new qPCR assay for the detection and characterisation of BFDV infection.

4.2 MATERIALS & METHODS

Insofar as was practical, the bELISA was developed and validated in accordance with guidelines established by the Office International des Epizooites (O.I.E.) (Belak and Thoren, 2004, Jacobson, 2004).

4.2.1 Samples

DNA extracts used for the development of the qPCR were taken from the archive of samples previously tested in our laboratory. Samples originated from the blood and feathers of many psittacine species, including sulphur-crested cockatoos (*Cacatua galerita*), galahs (*Eolophus roseicapillus*), rainbow lorikeets (*Trichoglossus haematodus*), purple crowned lorikeets (*Glossopsitta porphyrocephala*), eclectus parrots (*Eclectus roratus*), budgerigar (*Melopsittacus undulatus*), Indian ringneck (*Psittacula eupatria eupatria*), Jardine's parrot (*Poicephalus gulielmi*), green-cheeked conure (*Pyrrhura molinae*), sun conure (*Aratinga solstitialis*) and cockatiel (*Nymphicus hollandicus*).

To validate the qPCR, the DNA extracts of 108 blood samples from eastern long-billed corellas (*Cacatua tenuiostris*) vaccinated with a recombinant BFDV capsid protein (Bonne, Shearer, Sharp, Clark, Raidal unpublished 2008) and challenged with live virus were tested using the optimised qPCR assay. Crude DNA extracts from 108 feather eluates prepared for HA testing were also tested using the optimised qPCR assay.

Blood was collected by venepuncture of the jugular or cutaneous ulnar vein and spotted onto filter paper (Whatmann Grade No. 3), then allowed to 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.

4.2.2 Extraction of DNA from feathers

Viral DNA was extracted from feathers using the methods described by Ypelaar *et al* (1999). Five mm of feather calamus was cut on a sterile surface and placed into a microcentrifuge tube (Eppendorf). To this, 200 µL of 70% (v/v) ethanol were added and the tube vortexed briefly then the ethanol was removed and 200 µL of sterile distilled water were then added and the tube vortexed again. The sterile water was removed and 500 µL of lysis buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.0), 2.5 mM MgCl2, 0.005% (v/v) Tween 20, 0.005% (v/v) Nonidet P40], containing 250 µg/mL proteinase K (Qiagen) were added. The feather in lysis buffer was incubated at 37ºC for one to two hours, before being heated to 95ºC for 10 minutes. The solution was centrifuged and DNA was extracted from the supernatant with the Qiagen blood mini kit (Qiagen), using the blood and body fluid spin protocol.

Crude DNA extracts of feather eluates prepared for HA testing were also made. Feathers were incubated with 100 µL PBS at 60ºC for 1 hour in a microcentrifuge tube (Eppendorf). The solution was centrifuged briefly after incubation and 10 µL of the supernatant transferred to another microcentrifuge tube, then boiled for 10 minutes.

4.2.3 Extraction of DNA from dried blood spots:

Viral DNA was extracted from dried blood spots using Qiagen blood mini kit (Qiagen), using a modified dried blood spot protocol. Three spots of blood collected and dried on filter paper were cut out using scissors or a hole punch and collected in a microcentrifuge tube (Eppendorf). 180 µL of lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl2, 0.005% (v/v) Tween 20, 0.005% (v/v) Nonidet P40) were added in place of the Qiagen lysis buffer and the tube incubated at 85ºC for 10 minutes, then centrifuged. Twenty microlitres of proteinase K (Qiagen) were then added to the tube, the tube vortexed and then incubated at 56ºC for 1 hour. After incubation, the solution was centrifuged and DNA extracted from the supernatant according to the manufacturer's protocol.

4.2.4 Quantitative, real-time polymerase chain reaction assay

Primers were designed based on conserved regions of known BFDV sequences (Kumar *et al.,* 2004, Wishart and Fortin, 2001, Wishart *et al.,* 2000). Primers P5 (5'-GGA CGC AAA ATG AAG GAA G-3') and P6 (5'-TAG CGA GAG GTT ATG CAA GC-3') (Geneworks) were designed to amplify an 81bp fragment of ORF V1. Magnesium chloride concentrations and annealing temperatures were optimised using an Eppendorf Mastercycler Gradient

thermocycler. The optimised PCR reactions consisted of 2 mM $MgCl_2$, 5 µL of 5x polymerisation buffer containing dNTPs, 3.34 µM SYTO9 fluorescent dye (Invitrogen), 12.8 pmol of each primer and 0.1U of Tth Plus DNA polymerase, ultrapure water in a total volume of 23 µL (all reagents Fischer Biotec, except SYTO9), plus 2 µL of extracted DNA. Known-copy-number DNA standards were included in each run for quantitation of viral load.

Known-copy-number DNA standards were generated using various dilutions of a synthetic oligonucleotide, BFDV qST (5'-ATG TCC GGA CGC AAA ATG AAG GAA GTC GCA GCT GAA TTC CGA AAT TCC TAC GTC AGG CAT GGG CGT GGC TTG CAT AAC CTC TCG CTA TTG GTT-3') (Geneworks). The oligonucleotide was diluted to an initial concentration of $1x10^{12}$ copies/µL, then serially diluted 1:100 to give standards of $1x10^{10}$, $1x10^{8}$ and $1x10^{6}$. The $1x10^{6}$ standard was serially diluted 1:10 to give standards of $1x10^{5}$, $1x10^{4}$, $1x10^{3}$, $1x10^{2}$ and the $1x10^{2}$ standard diluted 1:2 to give a standard of 50 copies/µL. Also, 2 reactions using ultrapure water instead of DNA were used as negative controls in each run.

Reactions were carried out in a Corbett Rotor-gene 3000 (Corbett Research) real-time thermocycler. Cycling conditions consisted of an initial denaturation at 95ºC for 5 minutes, then 40 cycles of 95ºC for 20 seconds, 58ºC for 30 seconds and 72ºC for 20 seconds, followed by a final extension step at 72ºC for 10 minutes.

4.2.5 Melt curve analysis

A melt curve analysis was performed after each run, with the mixture being cooled to 60ºC for 45 seconds then heated in one-degree increments to 95ºC. Fluorescence was measured for 15 seconds at each increment and a graph plotting the second derivative of the melt curve displayed. The expected melting temperatures of the PCR products were between 80ºC and 85ºC; the melting temperature of the synthetic oligonucleotide was 82.3ºC.

4.2.6 Quantitation of viral load

The C_T values of the known-copy-number standards were graphed against time to construct a standard curve using the software supplied with the Rotor-Gene. The C_T of each of the samples was then compared against the graph to give an estimate of viral load.

4.3 RESULTS

4.3.1 A universal, quantitative polymerase chain assay for BFDV DNA

The qPCR assay successfully detected BFDV DNA in the DNA extracts of all known positive samples from all psittacine species tested.

The assay successfully detected BFDV DNA in the blood of all control (non-vaccinated) corellas (Figure 1a). In non-vaccinated control birds the viral load ± SE was estimated at 1 358 473 ± 1 113 226 copies/µL (range 14 478 to 5 768 973 copies/µL) at 2 weeks post

challenge and rose to a peak of 4 850 482 ± 4 775 008 copies/µL (range 1 709 and 23 949 983 copies/µL) by 4 weeks post challenge before dropping to 575 486 ± 551 069 copies/µL (range 823 and 2 779 419 copies/µL) at 6 weeks post challenge. Transient low-level viraemia of between 58 and 4 057 copies/µL was detected in 6 vaccinated birds at various time points, but all birds were seropositive at the times when viral DNA was present in blood samples. The viral load in blood samples of vaccinated birds followed a similar pattern to that of the non-vaccinated control birds, with mean viral loads increasing in a curvilinear fashion from 58 copies/µL at 2 weeks post challenge to a peak of 1177 ± 723 copies/µL (range 209 to 4 057 copies/µL) by 6 weeks post challenge (Figure 1a). Specific peaks were present in the melt curves of BFDV-positive samples between 82 – 84.5ºC.

Intra-assay precision was good, with r-squared values for the standard curves achieving values between 0.92693 and 0.97477. Inter-assay variation of the standards was only fair, as the mean coefficient of variation of the standards was 37.16%. The limit of detection (LOD) of the assay was 50 copies/µL, however the effective limit of quantitation (LOQ) was 1000 copies/µL, as the coefficient of variation of the 10^3 and 10^2 standards was close to 1 and as such was considered too high. Even though the qPCR assay successfully detected as few as 50 copies/µL of the standard, calculated quantities for the "50 copies/µL" standard varied between 50 and 625 copies/µL.

The assay also detected BFDV DNA in the crude DNA extracts of HA feather preparations. The mean viral load ± SE in feather extracts of control birds was estimated at 8 831 ± 4 662 (range 262 to 20 639 copies/µL) at 2 weeks post challenge, rose to a peak of 852 500 308 ± 681 941 583 copies/µL (range 2 591 to 3 409 766 576 copies/µL) at 4 weeks post-challenge, then decreased to 380 734 071 ± 380 729 701 copies/µL (range 430 to 1 903 652 876 copies/µL) by 6 weeks post challenge. Virus was detectable by HA in the feather samples of only one control bird at 4 and 6 weeks post challenge, which were also the samples that had the greatest amount of viral DNA present. Viral DNA was present in the feather extracts of 3 vaccinated birds 2 weeks post-challenge, 6 vaccinated birds at 4 weeks post-challenge and 10 vaccinated birds 6 weeks post-challenge. However, none of these 3 birds had detectable viral DNA in the equivalent blood sample at 2 weeks post-challenge. At 4 and 6 weeks post challenge, only 1 of 6 and 4 of 10 birds respectively had detectable amounts of viral DNA in the equivalent blood sample (between 193 and 420 451 copies/µL). The samples which were positive on qPCR of feather extracts but negative on blood samples were considered to be false positives. Correlation of viral load between blood and feather samples was moderate (r^2 = 0.7465, $p<0.05$) at 2 weeks post challenge and very high (r^2 = 0.9999) at 4 weeks post challenge, but there was no significant correlation at 3 and 6 weeks post challenge (r^2 = 0.0048 and 0.0027, respectively).

Two sets of comparisons were made for the calculation of sensitivity and specificity. Firstly, similar tests were compared (standard PCR with qPCR using blood samples and HA with qPCR using HA samples). Sensitivity was 94.73% and specificity 87.61%, with positive and negative predictive values of 56.25% and 99% respectively, when the qPCR using blood samples was compared to the standard PCR (also performed on blood samples). When comparing the qPCR using HA samples to HA test results, sensitivity was 100% and specificity 65.42%, the positive predictive value dropped to 5.13% and the negative predictive value was 100%. Secondly, dissimilar tests were compared (standard PCR with qPCR using HA samples and HA with qPCR using blood samples). Comparing qPCR results from blood

samples to HA results gave a sensitivity and specificity of 100% and 71.43%, respectively, while comparing qPCR results from HA samples with standard PCR results gave a sensitivity and specificity of 72.22% and 61.43%, respectively.

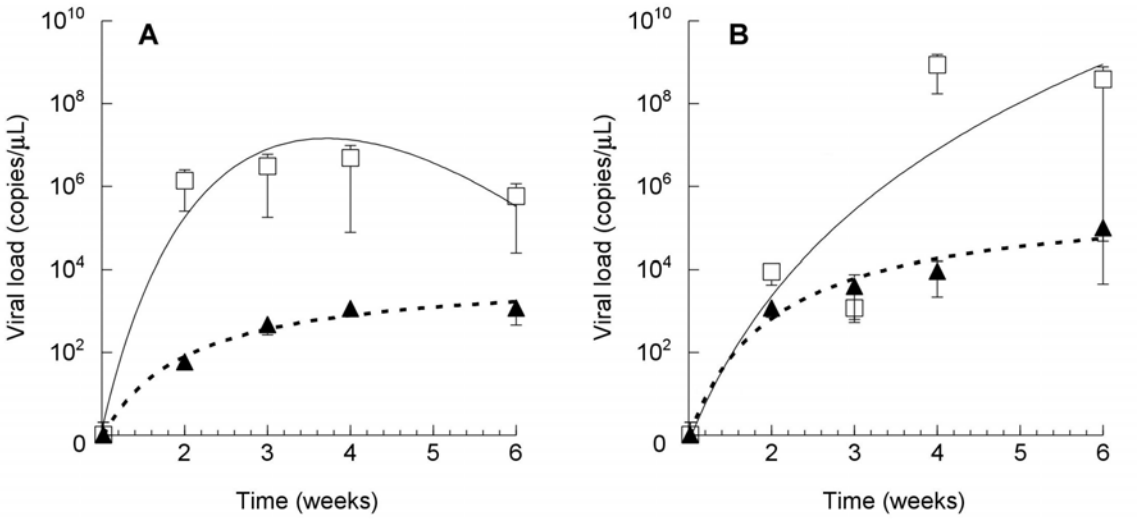

Figure 12. Mean viral load in A: blood and B: feather preparations of control (□) and vaccinated birds (▲) after challenge with live BFDV. Peak viral load was greater in feather preparations than in blood samples for both groups.

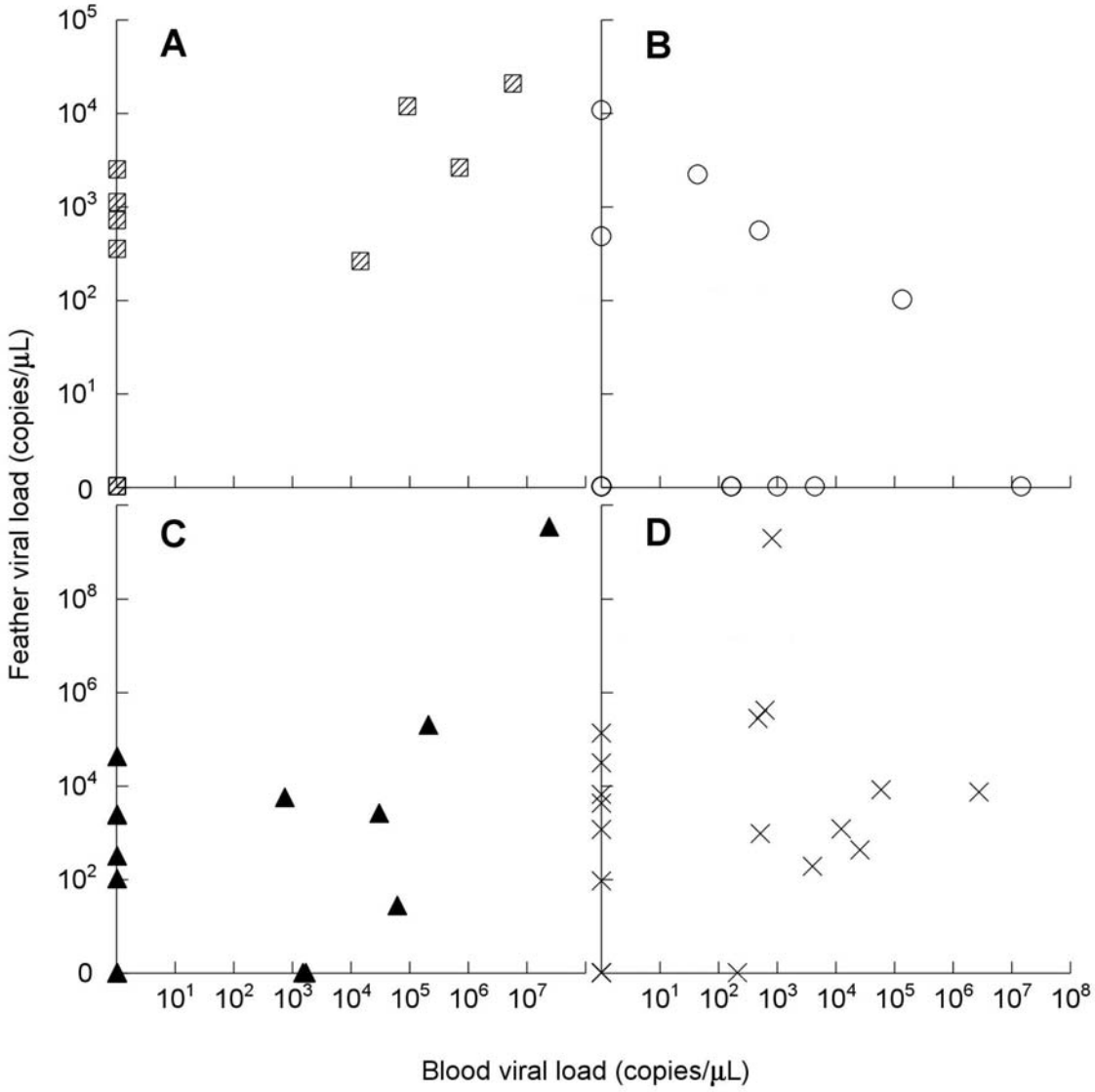

Figure 13. Correlation between blood and feather viral loads of both vaccinated and control birds. A: 2 weeks post-challenge, B: 3 weeks post challenge, C: 4 weeks post challenge, D: 6 weeks post challenge. Values were highly correlated at 4 weeks post challenge, but did not correlate at 3 and 6 weeks post challenge.

4.4 DISCUSSION

Given the high prevalence of BFDV infection worldwide, readily standardised, sensitive and specific detection methods are both highly desirable and necessary. The qPCR assay described here is extremely sensitive and can be used to quantify the amount of virus present in the blood and in feather preparations. As such, it should prove to be a useful assay for the detection of viral DNA in samples of blood, tissues, feathers and faeces. Only one other real-time PCR assay for BFDV has been described (Raue *et al.,* 2004). This assay was not quantitative and tested only 31 samples, however it could easily be modified to a quantitative assay using the standards described by the authors.

The analytical sensitivity of the qPCR assay was greater than that of the standard PCR assay, as it detected viral DNA in the blood of control birds that were PCR negative. However, because of this fact the analytical sensitivity could not be determined precisely. The comparative diagnostic sensitivity and specificity was good, however because of the greater analytical sensitivity of the bELISA, the absolute diagnostic sensitivity and specificity is likely to be higher. Given the calculated diagnostic sensitivity, 542 samples would be required to validate the assay with 95% confidence of an accurate result (Jacobson, 1998). Assuming the highest calculated diagnostic specificity of 88%, the number of known negative animals required is 1 014. Only 166 samples were available from birds of known infection status, and even though the actual diagnostic sensitivity is likely to be higher (and hence less samples required) ongoing testing is always recommended (Belak and Thoren, 2004, Jacobson, 2004). The precision and repeatability of the assay are good; reproducibility could not be assessed since testing in other laboratories was impractical. Negative predictive values were uniformly good and the low positive predictive value when comparing the PCR and qPCR assays on blood samples is likely to be a result of the increased sensitivity of the assay. When comparing the HA assay with qPCR assay performed on feather samples, the extremely low positive predictive value reflects the large number of false positives as a result of environmental contamination. Given the high prevalence of BFDV, the assay should be useful for testing samples from naturally infected birds, appropriate samples are collected and properly stored and processed.

The advantage of the qPCR assay over non-quantitative methods for the detection of viral DNA is highlighted in this study and the vaccination trial of which it is a part (Bonne *et al*, 2008). Not only did the qPCR assay detect viral DNA in birds that tested negative by the standard PCR (Bonne, Shearer, Sharp, Clark, Raidal unpublished 2008), but it demonstrated that the viral load in both vaccinated and control birds increased for the first 4 weeks after challenge. That this occurred despite vaccinated birds having anti-BFDV antibodies before being challenged (Bonne, Shearer, Sharp, Clark, Raidal unpublished 2008) indicates that vaccination does not prevent viral replication. This is a common scenario with other vaccines (Opriessnig *et al.,* 2006) and is not surprising in this case. This evidence of viral replication would not have been detected if a standard PCR assay alone had been used. Given that vaccination does not prevent viral replication, it is likely that the chicks of vaccinated birds may still be infected despite the presence of antibodies in the parents, as occurs with PCV2 and CAV (Brentano *et al.,* 2005, Larochelle *et al.,* 2000). This mode of infection is more likely to be significant for BFDV than PCV or CAV, as psittacine birds live much longer than pigs or chickens. If this does occur, the clinical and molecular significance of such infections and the impact of this mode of transmission on quarantine and hygiene programs would need to be thoroughly investigated.

The main advantages that this qPCR assay has over existing PCR assays is the ability to estimate the amount of pathogen present in the sample and that post-reaction processing is not required to visualise reaction products. An absolute method of quantitation was selected for this assay, as it was useful to assign a set quantity to measurements. Notably, though, no quantitation method is foolproof and both absolute and relative quantitation methods are both legitimate, provided that the assay itself is properly validated. Peirson *et al* (2003) found that results using the two absolute and three relative methods of quantitation they evaluated compared very favourably. Relative quantitation methods have the distinct advantage over absolute quantitation methods in that artificial standards are not required; the cumulative error

induced by spectrophotometry, molecular weight calculations and pipetting errors can make calculations of absolute copy numbers meaningless. This cumulative error is likely to be responsible for the moderate inter-assay coefficient of variation. Provided that this variation is minimised and the assay properly validated, absolute quantitation can provide results in units (usually copies/µL or genome equivalents) that are common to both scientists and clinicians (Mackay *et al.,* 2002). The preparation of a "master batch" of standards and storage of these in pre-measured aliquots would likely go a long way to further ensuring consistency in this and other similar assays. In this assay, as few as 50 copies/µL could be detected, similar to the assay described by Raue *et al* (2004), however the actual LOD in that assay is unknown as the authors relied on dilution factors rather than quantitative data. The LOQ of this assay was determined to be 1000 copies/µL. The distinction between LOD and LOQ is important, as clinical interpretations made from samples with positive results below the limit of quantitation may be inaccurate.

Many qPCR assays normalise the data from the target gene or organism against one or more genomic controls (Hendriks-Balk *et al.,* 2007). The data in this study was not normalised to a reference (or housekeeping) gene as candidate genes for normalisation of gene expression data in psittacine birds have not been evaluated. Normalisation of data against control genes is necessary if the results of a qPCR assay are to be meaningfully compared between sample types and quantities, individual test subjects, times and testing laboratories (Tricarico *et al.,* 2002). For qPCR assays detecting microbiological agents, unless the pathogen is being grown in cell culture the real value of normalisation is that the normalised data can account for differences in sample quantity and quality. The selection of the control genes is important, as the levels of even commonly used genes can vary in different disease states, or isoforms of the gene may exist (Sturzenbaum and Kille, 2001). The optimal gene/s to use will also depend on the tissue sampled (Tricarico *et al.,* 2002), the disease state (Li *et al.,* 2005, Radonić *et al.,* 2005) and the host organism (Maccoux *et al.,* 2007, Robinson *et al.,* 2007, Sellars *et al.,* 2007, Spinsanti *et al.,* 2006, Tang *et al.,* 2007).

There have been surprisingly few studies investigating the suitability of avian reference genes (Li *et al.,* 2005, Yamashita *et al.,* 2007). As such, any BFDV qPCR assay that uses control genes for the normalisation of data should first thoroughly investigate the variability of these genes in the various tissues sampled and in both normal and BFDV-infected birds. The evaluation of suitable reference genes for use in this assay would certainly be an advantage, as feathers used for BFDV PCR assays typically vary in size and the quality of pulp material between growing and mature feathers varies dramatically.

Because of the high degree of genetic variation between BFDV isolates (Bassami *et al.,* 2001, de Kloet and de Kloet, 2004, Heath *et al.,* 2004, Kondiah *et al.,* 2006, Ritchie *et al.,* 2003) intercalating dye chemistries are the method of choice for the detection of BFDV DNA in quantitative PCR assays. SYTO9, rather than SYBR green, was chosen for use in this assay as the melting curves are highly reproducible over a greater range than SYBR green, it has less of an inhibitory effect on the PCR reaction and does not appear to selectively detect particular amplicons (Monis *et al.,* 2005).

The qPCR assay described here has proven to be useful for the detection of BFDV DNA in blood samples, but the high sensitivity of the assay makes the use of HA feather preparations unreliable for the detection and quantitation of viral excretion. Firstly, as with HA testing, the

difficulty in ensuring consistency in the amount of material tested makes standardisation problematic. Secondly, false positive results were reasonably common. The increased incidence of false positive results from feather preparations over time was almost certainly due to sample contamination via aerosolised feather dander and faecal material. The shape of the graph of viral load in feather extracts of vaccinated birds (Figure 11b) is similar to those of viral load in blood samples of vaccinated and non-vaccinated birds (Figure 11a), which suggests viral replication. However, this pattern more likely reflects the increased viral load in the environment as a result of virus excretion by non-vaccinated birds. Choanal/cloacal swabs will most likely be more useful than feather suspensions for the detection and quantitation of excreted virus, but each new sample type should be properly validated before use in routine diagnostic testing (Hoorfar *et al.,* 2004). Cloacal swabs have been found to be useful for the detection of pigeon circovirus (Todd *et al.,* 2006), however no firm conclusions on their usefulness can be drawn since the technique was not fully validated. Storage of swabs will be important if environmental contamination is to be avoided; swabs which are left unshielded before and after sampling will likely come into contact with feather dander and/or faeces from BFDV-infected birds, thus rendering the test useless.

Potential applications of this assay include the measurement of viral load in many tissues (including formalin-fixed, paraffin-embedded tissues) and from cloacal swabs, as well as the development of multiplexed assays for the detection of common psittacine pathogens (e.g. *Chlamydophila psittaci*, *Macrorhabdus ornithogaster* and *avian polyomavirus*). The development of quantitative multiplex PCR assays that include the quantitation of BFDV DNA should first thoroughly evaluate the effect of primer and probe mismatches due to the variability of the BFDV genome. Alternately, a qualitative (quantal) multiplex assay could be developed more simply using an intercalating dye, in which the presence of the pathogens is indicated by the melting peaks of the PCR product. A good example of a similar technique is the development of a standard multiplex PCR for the detection of PiCV, *pigeon herpesvirus* (PiHV) and *fowl adenovirus* (FAdV) causing young pigeon disease syndrome (YPDS) (Freick *et al.,* 2008). Furthermore, if infectivity bioassays are conducted to determine the ID50 of BFDV and the proportion of detected virus that is infectious, as has been done for HIV-1 (Coombs *et al.,* 1993, Rusert *et al.,* 2004), then the qPCR assay could be used to determine the amount of infectious virus in a sample.

At present, the haemagglutination and haemagglutination-inhibition assays are commonly used for the detection of virus shed from feathers or faeces and anti-BFDV antibodies in psittacine sera, respectively. They require erythrocytes from live animals and virus purified from the feathers of infected birds. Polyclonal antibody preparations and variations in these reagents make consistency between tests difficult to achieve. Consequently, most laboratories rely on PCR-based assays to test for the presence of viral DNA in blood, faeces or feathers, but the lack of consistent and reliable assays for serology and the detection of excreted virus is a problem. PCR assays can also vary in sensitivity and specificity between laboratories (East *et al.,* 2004) and are susceptible to contamination (Bonne *et al.,* 2007) and inhibition (Knutsson *et al.,* 2004). Variation in erythrocyte sensitivities between avian species and individual birds (Sanada and Sanada, 2000, Sexton *et al.,* 1994) has been cited as a problem in the standardisation (Johne *et al.,* 2004) of HA and HI assays. However, in regards to PCR assays it must be remembered that poor sample collection and quality, inter-laboratory variation, improper attention to the prevention of contamination and assay-based factors (such as pipetting error, reagent quality and the presence of inhibitors) can render a test

diagnostically and clinically useless. The susceptibility to contamination of such sensitive assays is highlighted in this study.

Reliance on PCR-based diagnostic tests can also lead to false clinical assumptions since the presence of viral DNA does not necessarily indicate active infection. Non-replicating DNA may be present in blood for up to 3 months (Lazizi and Pillot, 1993) and dead bacteria may continue to be excreted for 15 days after successful therapy (Gaydos *et al.,* 1998). Additionally, seropositive birds may still be PCR positive whilst they are clearing the virus (Khalesi *et al.,* 2005) and testing of these birds by PCR alone would miss this important piece of information. Nonetheless, provided these factors are considered, the qPCR assay described here should prove to be a sensitive, specific and accurate diagnostic tool for the detection and characterisation of BFDV infection.

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Chapter 5. General Conclusions

This project has produced high quality synthetic BFDV capsid protein that is antigenic and capable of self assemblage into virus like proteins. Its use to produce monoclonal antibodies against BFDV also assisted with the development a blocking ELISA assay which has the potential to replace haemagglutination inhibition as the gold standard serological assay for BFDV infection. The project also developed a quantitative PCR assay for the sensitive and quantitative detection of BFDV DNA in diagnostic samples. These new assays will greatly enhance the ability of diagnosticians to determine the infection and disease status of psittacine birds and flocks. A study comparing the relative merits of antigen, antibody and viral DNA detection demonstrated that all three modalities need to be applied to obtain meaningful results for individual birds. Also the results of such testing must be interpreted alongside other information such as the species, age, sex and clinical signs exhibited by the bird. Experienced avian veterinarians are the best qualified scientists to interpret diagnostic assay results on an individual bird basis.

5.1 RECOMMENDATIONS

The development of baculovirus recombinant BFDV protein is a major achievement because it has solved the problem of producing high quality BFDV antigen that can be used for diagnostic assays and as a candidate vaccine. There is a high priority to employ this technology to synthesise sufficient capsid protein both for the bELISA assay and for its use as a vaccine. There is also a need to ensure continuity of antigen production if it is to be used for these purposes. This action should be taken quickly to avoid having to reinvent the method. Furthermore before this recombinant protein can be used to vaccinate endangered species, further research investigations are required as follows:

5.1.1 Developing an efficient method to increase the volume of production of recombinant baculovirus capsid protein.

This is a high priority so that efforts so far made are not lost. The diagnostic technologies have been developed and now they should be used. But to do that it is important to have a supply of reagents and an expert laboratory technician who can perform the assays.

5.1.2 Determining whether it will be necessary to include purification steps in the production of the recombinant capsid protein.

The level of purity that is required for the recombinant BFDV protein is important because it may well be that impure antigen is more immunogenic than highly purified antigen. This is especially true for vaccine development. Purification processes always reduce the yield of antigen and may not be absolutely necessary.

5.1.3 Vaccination trials in a range of small to large psittacine bird species.

So far most of the BFDV vaccine research has used cockatoos and it may well be the case that information generated in this group of birds cannot be applied to smaller species such as the *Neophema*. It is therefore required to determine the minimal effective vaccine dose of recombinant protein that will induce a persistent effective immunity in a range of different bird species and of different ages and physiological status. This will include determining the number of doses required and the optimal time between doses.

5.1.4 Adjuvant safety

Adjuvants are chemicals used in the manufacture of killed vaccines which have qualities that stimulate the immune reaction. Many work by being irritant and thus causing an inflammatory reaction which can have adverse consequences to vaccinated birds. It is therefore important in any vaccine development to ensure that the most appropriate and safe adjuvant is selected that retains high immunogenicity. This is especially important for a vaccine that might be used in a critically endangered species.

5.1.5 A better understanding of the antigenic variation that exists among BFDV isolates.

We still do not know very much about BFDV isolates from wild endangered psittacine bird species which will also be required to ensure that any vaccine developed is efficacious against all antigenic "serotypes". This is due to our recent discovery of antigenic variation in cockatiel BFDV isolates.

5.1.6 Vaccination and challenge experiments

Experimentally vaccinated birds have to be challenged with live virus to determine whether vaccination confers immunity to BFDV infection. This will require the development of specific pathogen free flocks (SPF) of psittacine birds that can act as experimental models of infection. It will be necessary to determine the level of passively transferred immunity that occurs between parent (hen) and offspring via the egg yolk which could both facilitate protection of nestling birds but also interfere with vaccination of progeny.

5.1.7 Novel vaccination strategies for free-flying wild birds

Future development of novel techniques that can facilitate the safe and efficacious vaccination of free-living wild birds with minimum impact on individual birds and the environment should also be considered. This could include DNA vaccines that could be applied at feeding stations or directly into nest hollows without the need to capture, handle and inoculate birds.

5.1.8 Cost Estimates of future work required

Future work required					
Action	Priority	**Indicative cost estimate**			
		2008	2009	2010	2011
Increasing production volume and purification of recombinant protein for use in vaccination and diagnostic assay	High	$30,000*	$130,000	$95,000	$95,000
Improved understanding of the antigenic and genotypic variation of BFDV, particularly in endangered birds	Medium		$20,000	$20,000	$20,000
Develop an SPF flock that can be used for vaccination response and protection experiments	High		$100,000	$100,000	$100,000
Vaccination dose, challenge and effectiveness experiments	High		$80,000	$80,000	$80,000
Development of novel "minimum interference" techniques for administering vaccine to wild birds	Low			$90,000	$90,000
Disease modelling	High		$20,000	$20,000	$20,000

*Continuity and transfer of project to NSW