Development of Novel Diagnostic and Vaccine Options for Beak and Feather Disease Virus (BFDV)

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This thesis is presented for the degree of Doctor of Philosophy at Murdoch University September 2008
I declare that this is my own account of my research, except where duly acknowledged and contains work that has not been submitted for a degree at any tertiary institution

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Abstract

Beak and Feather Disease Virus (BFDV) is a circovirus which causes ill-thrift, feather loss and immunosuppression leading to secondary infections and eventually death in psittacine birds. The development of standardised reagents for the detection and characterisation of BFDV infections and for the production of protective vaccines has been difficult as no cell culture system has yet been found to grow the virus successfully in vitro. However, the development of consistent and effective diagnostic tests and vaccines is now more practical through the application of nucleic acid-based detection methods and recombinant technology.

A quantitative real-time PCR assay for the detection of BFDV DNA was developed, using primers designed to amplify a conserved 81 bp fragment of ORFV1 and SYTO9, a fluorescent intercalating dye, with assays run on a Corbett RotorGene 3000. A synthetic oligonucleotide was used to establish standard curves for the quantitation of viral load in both blood and feather preparations. The assay was very sensitive, with a detection limit of 50 copies/µL. The assay was developed using BFDV-positive DNA extracts from the feathers of 10 different species of birds and validated with blood and feather samples from corellas vaccinated with an experimental BFDV vaccine, then challenged with live virus. Viral DNA was reliably detected in the blood of all control (non-vaccinated) birds and in some vaccinated birds. Contamination of the environment with the feather dander of BFDV-infected birds meant that HA feather preparations were unreliable for the detection and quantitation of viral excretion. Nonetheless, the assay should prove to be a useful and sensitive test for the detection of viral DNA in a range of samples in future investigations.
A recombinant BFDV capsid protein was also produced and a specific monoclonal antibody developed against it. The behaviour of the protein in haemagglutination (HA) assays and the behaviour of the monoclonal antibody in western blotting, immunohistochemistry (IHC), ELISA and haemagglutination-inhibition (HI) assays were characterised. The protein had the ability to agglutinate galah erythrocytes as per the wild-type virus and this agglutination was successfully inhibited by antibodies to wild-type BFDV from naturally immune psittacine birds. Furthermore, the protein self-assembled into virus-like particles as determined by electron microscopy. The antibody was specific for both the recombinant BFDV capsid protein and the whole virus and had similar optimal titres when used in western blotting and IHC. The antibody also had HI activity and detected BFDV virus from 3 genera of psittacine birds, including the recently described cockatiel BFDV isolate. A novel “blocking” (or “competitive”) ELISA (bELISA) for the detection of anti-BFDV antibodies in psittacine sera (Ab-bELISA) was also developed and validated with 166 samples from eastern long-billed corellas vaccinated with the recombinant capsid protein and challenged with live virus. The bELISA was found to be both sensitive and specific and correlated strongly with the HI test, thus it should have wide application for the serodiagnosis of BFDV.

A survey of cockatiels (n=88) housed at commercial aviaries was conducted to investigate whether BFDV infection occurs in cockatiels. All birds were diagnosed as being virus-free by PCR and HA and had no detectable antibody titre by HI assay. In addition to this, the genomes of two BFDV isolates obtained from diseased cockatiel feathers were sequenced and cross-reactivity assays performed using virus eluted from these feathers and sera from naturally immune psittacine birds. Serological
cross-reactivity results and phylogenetic analysis of the nucleotide sequences indicated that the cockatiel virus isolates were serologically and genetically different to other BFDV isolates. This is the first report of an antigenically distinct BFDV in psittacine birds. Since the Ab-bELISA has a lower limit of detection than the HI assay, it was used to repeat the cockatiel sero-survey. No antibodies were detectable in any of the cockatiels tested and thus questions about the real prevalence of BFDV infection in cockatiels and the possible existence of a novel BFDV serotype adapted to cockatiels remain unanswered.

The successful control of PBFD in both pet and wild birds depends on the development of vaccines that incite a strong specific immune response and can be efficiently produced in large quantities. Recombinant BFDV capsid proteins have recently been considered as candidate vaccines against BFDV and recombinant techniques allow the development of other candidate vaccines, including DNA vaccines. In order to examine the potential of DNA vaccination as a strategy for the prevention and control of BFDV, two DNA vaccines, based on the nucleotide sequence encoding the capsid protein of BFDV, were developed using the mammalian expression vector pVAX1. The vaccine constructs encoding both the full-length and NLS-truncated capsid protein resulted in protein expression both in vitro and in vivo. Protein was detected in COS-7 cells transfected with the constructs with an indirect immunocytochemistry assay using the monoclonal antibody described in Chapter 5. Protein was present in the nucleus of cells transfected with the vaccine encoding the full-length nucleotide sequence and in the cytoplasm of cells transfected with the vaccine encoding the NLS-truncated sequence as expected. Both DNA vaccine constructs induced detectable levels of anti-BFDV antibodies in vaccinated birds, determined using the Ab-bELISA described in Chapter 5. Thus,
DNA vaccines similar to those presented here may have application in the prevention and control of BFDV and some options for the further development of these vaccines into effective methods for the control of BFDV are discussed.
Acknowledgements

There are so many people I would like to thanks for their help throughout my PhD, so here goes...

My supervisors, Shane Raidal and Phil Clark have been everything I wanted from supervisors and more. Thanks for letting me pursue my own ideas and reigning me in when I got carried away, thanks for your thorough attention to my work and for getting things back to me in record time, and most of all thanks for letting me drop in and chat whenever I felt like it (no matter how degenerate the conversation became). Thanks especially to Shane for keeping up with supervision despite being over the other side of the country and to Phil for taking me (and many others) under his wing when Shane left. Phil went above and beyond the call of duty looking after Nico and myself after leaving Murdoch.

A big shout out to Nico, my co-conspirator. You’ve been a huge help all the way through the process, from translating molecular biology jargon to trading dirty jokes. The vaccination trial was also conducted in collaboration with Nicolai and he performed the HA, HI and standard PCR assays for it. Huge hugs to Marg and Clare for taking me under their wing (no pun intended) and showing me how to do things properly. Marg, I think you’ve pretty much accomplished the almost impossible task of turning a vet into a scientist! To Ingrid as well, we couldn’t have dealt with all those baby birds without you, thanks for being part of the team. Of course we can’t forget the members of Feathers, Fur and Filter-Feeders Inc. – Doug, Zoe, Mark, Lisa, Lucy, Tim (Tim’s an honorary member). Without you guys to trade ideas and rude emails with I would never have survived academic life at Murdoch.
I owe many people thanks for their fantastic advice and technical help and for providing materials as well. I think of my thesis as a collaborative effort and it could not have happened without you. Thanks go to: Meredith for developing most of the baculo-protein system and for the benefit of her unique thought processes; Linda Davies for taking care of so much of our insect cell cultures; Andrew and Mark for helping us out with troubleshooting protein production and swapping protein samples with us; Dr Kathleen Davern, Tamara Jacoby and the rest of the WA Monoclonal Antibody Facility for holding my hand through the process of producing the monoclonal antibody; Dr Andrew Mikosza for making qPCR work seem a lot less scary; Dr Rob Messing from Proteomics International for helping me to work out how much protein we actually had; Dr Will Ditcham for helping me work out how to make a DNA vaccine and for giving me some pVAX cultures; Assoc. Prof Cassie James and Josie for helping me to understand what a DNA vaccine is and for giving me the COS-7 cells and finally Mike Slaven and Gerard Spolestra for all their help with my histology and IHC work.

My family and friends have been there every step of the way, offering advice, distractions and free meals. Thanks Mum for fussing over me, Dad for all the ideas and encouragement and Cath for talks about life, the universe and everything. Terrence and Krissi, thanks for the travels, the frequent Chutney Mary’s picnics and for just hanging out and bitching about the uni.

Last, but certainly not least, to Danielle for everything she’s done for me throughout the thesis. From putting up with me when lab work ran late and helping to raise the baby birds to spending all our free time on travels and adventures. Love you lots, and lots. And lots:)
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List of Units

bp       base pair
cm       centimetre
Da       daltons
°C       degrees celsius
g       gram
g/L     grams per litre
g/mL    grams per millilitre
xg      times gravity
h       hours
HAU      haemagglutination units
HIU      haemagglutination inhibition units
kb       kilobase
kDa      kilodaltons
L       litre
mM      millimolar
M       molar
min     minutes
mL      millilitre
ng      nanograms
ng/µL   nanograms per microlitre
nm      nanometre
nt      nucleotide
pfu/mL  plaque-forming units per millilitre
pmol    picomole
rpm     revolutions per minute
s       seconds
TCID₅₀/mL median cell culture infectious dose per millilitre
U       units
µg/mL   micrograms per millilitre
µL      microlitre
µm      micrometre/micron
µM      micromolar
U/mL    Units per millilitre
V       volts
v/v     volume per volume
w/v     weight per volume
Abbreviations

Ab-bELISA antibody blocking ELISA
ABTS 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
Abs absorbance
APC/C anaphase promoting complex/cyclosome
APV avian polyomavirus
BBTV banana bunchy-top virus
BFDV beak and feather disease virus
BCTV beet curly top virus
BSA bovine serum albumin
cap/Cap capsid gene/protein
CAV chicken anaemia virus
CaCV canary circovirus
CFDV coconut foliar decay virus
Ci-TTV canine torque teno virus
CIA chicken infectious anaemia
COS-7 simian immunodeficiency virus-transformed African green monkey kidney cells
CPE cytopathic effect
CsCl caesium chloride
CT threshold cycle
DAB diaminobenzidine
DMEM Dulbecco’s modified Eagle’s medium
DNA deoxyribonucleic acid
DNase deoxyribonuclease
dNTP deoxynucleotide triphosphate
dsDNA double stranded DNA
dTTP deoxythymidine triphosphate
DuCV duck circovirus
EDTA ethylenediaminetetraacetic acid
ELISA enzyme-linked immunosorbent assay
EM electron microscope/microscopy
FAdV fowl adenovirus
FBNYV faba bean necrotic yellow virus
FCS foetal calf serum
Fc-TTV feline torque teno virus
GoCV goose circovirus
HA haemagglutination
HAU haemagglutination units
HBV hepatitis B virus
HCl hydrogen chloride
HI haemagglutination-inhibition
HRP horseradish peroxidase
ICC immunocytochemistry
ID$_{50}$ median infectious dose
IFN-α alpha interferon
IFN-γ gamma interferon
IHC immunohistochemistry
IL interleukin
IM intramuscular
IPTG isopropyl-p-D-thiogalactopyranoside
ISH in-situ hybridisation
KCl potassium chloride
LOD limit of detection
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>LOQ</td>
<td>limit of quantitation</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption/ionisation time of flight</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MSV</td>
<td>maize streak virus</td>
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<td>NES</td>
<td>nuclear export signal</td>
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<td>NIPC</td>
<td>natural interferon producing cell</td>
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<td>NLS</td>
<td>nuclear localisation signal</td>
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<td>NPC</td>
<td>nuclear pore complex</td>
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<td>ODN</td>
<td>oligodeoxynucleotide</td>
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<tr>
<td>OIE</td>
<td>office international des epizooties</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>PBFBD</td>
<td>psittacine beak and feather disease</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PBST</td>
<td>PBS plus 0.05% (v/v) Tween 20</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PCV</td>
<td>porcine circovirus</td>
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<td>PDNS</td>
<td>porcine dermatitis and nephropathy syndrome</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
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<td>PI</td>
<td>percentage inhibition</td>
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<tr>
<td>PiCV</td>
<td>pigeon circovirus</td>
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<tr>
<td>PiHV</td>
<td>pigeon herpesvirus</td>
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<td>PMWS</td>
<td>postweaning multisystemic wasting syndrome</td>
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<tr>
<td>PMSF</td>
<td>phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>PO</td>
<td>per os</td>
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<td>PTA</td>
<td>phosphotungstic acid</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<td>RaCV</td>
<td>raven circovirus</td>
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<tr>
<td>RCA</td>
<td>multiply-primed rolling-circle amplification</td>
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<tr>
<td>RCR</td>
<td>rolling-circle replication</td>
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<tr>
<td>rep/Rep</td>
<td>replication-associated gene/protein</td>
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<td>RF</td>
<td>replicative form</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RNAi</td>
<td>ribonucleic acid interference</td>
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<tr>
<td>SCSV</td>
<td>subterraneanan clover stunt virus</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate - polyacrylamide gel electrophoresis</td>
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<tr>
<td>Sd-TTV</td>
<td>swine torque teno virus</td>
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<tr>
<td>SFM</td>
<td>serum-free medium</td>
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<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
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<tr>
<td>StCV</td>
<td>starling circovirus</td>
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<td>Tbc-TTV</td>
<td>tupaia torque teno virus</td>
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<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>TBST</td>
<td>TBS plus 0.05% (v/v) Tween 20</td>
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<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median tissue culture infectious dose</td>
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<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>TTV</td>
<td>torque teno virus</td>
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<tr>
<td>TLMV</td>
<td>TT-like mini virus</td>
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<tr>
<td>TYLCV</td>
<td>tomato yellow leaf curl virus</td>
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<tr>
<td>VLP</td>
<td>virus-like particle</td>
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<tr>
<td>VN</td>
<td>virus neutralisation</td>
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<tr>
<td>YPDS</td>
<td>young pigeon disease syndrome</td>
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Communications

Publications arising from this thesis


*Oral presentations at scientific meetings*


Shearer, P., Bonne, N., Clark, P., Sharp, M., Raidal, S. (2007). Cockatiels have their own beak and feather disease virus, August, Melbourne, AUSTRALIA
Chapter 1. General Introduction

Psittacine Beak and Feather Disease (PBFD) is the most common viral disease of psittacine birds and causes either: a chronic debilitating feather disease, typically in adult birds (Albertyn et al., 2004, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989b), or a severe, acute disease syndrome in nestlings and African grey parrots (*Psittacus e. erithacus*) (Doneley, 2003, Raidal and Cross, 1995, Schoemaker et al., 2000). The causative agent, beak and feather disease virus (BFDV), is a circovirus with a single stranded DNA genome, approximately 1.7-2.0 kb in length (Bassami et al., 2001, Ritchie et al., 1989b) and is considered to have a worldwide distribution (Albertyn et al., 2004, Hsu et al., 2006, Kiatipattanasakul-Banlunara et al., 2002, Kock et al., 1993, McOrist et al., 1984, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989a).

The development of diagnostic assays and vaccines for the detection and prevention of BFDV have traditionally been hampered by the difficulty associated with producing suitable reagents, namely purified virus and polyclonal antibodies. At present, haemagglutination (HA) and haemagglutination-inhibition (HI) assays (Raidal et al., 1993c, Ritchie et al., 1991b) are commonly used for the detection of virus shed from feathers or faeces and anti-BFDV antibodies in psittacine sera, respectively. These tests, whilst useful and applicable to samples from a wide range of psittacine birds, are not ideal assays; they require erythrocytes from live animals, virus purified from the feathers of infected birds and polyclonal antibody preparations in order to perform the assays. Variations in these reagents make consistency between tests difficult to achieve, underscoring the need for new tests with standardised reagents. The HI assay (Raidal et al., 1993c, Ritchie et al., 1991b) is so far the only method
available for the detection of anti-BFDV antibodies in psittacine sera. This assay is useful in that it detects both IgM and IgG 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 to the virus of the erythrocytes used in the test.

A killed whole-virus vaccine has been developed previously (Raidal and Cross, 1994a, Raidal et al., 1993a), but the antigen was purified from the feathers of 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. Incomplete inactivation of the virus may also result in clinical disease in vaccinated birds (Raidal et al., 1993a, Wylie and Pass, 1987). New diagnostic tests and vaccines, based on reagents which are easily standardised and able to be produced in large quantities, are therefore both desirable and necessary.

As a background to the research presented in this thesis, a review of the literature is presented in Chapter 2. Particular attention is paid to the replication and pathogenesis of circoviruses and phylogenetic analyses of various BFDV isolates. The volume of research on geminiviruses and porcine circovirus 1 and 2 is much greater than that of BFDV because these viruses are more economically important and a cell culture system has not yet been developed to successfully grow BFDV in vitro. As such, the discussion of circovirus replication presented here relies heavily on scientific studies of these other circoviruses. The second part of the discussion presents the current body of scientific knowledge on BFDV. The physical, chemical and molecular characteristics of the virus are summarised and the current thinking
on the phylogenetic relationships of various virus isolates and pathologic mechanisms are discussed.

Various polymerase chain reaction (PCR) assays have been developed for the detection of BFDV, however none of these are quantitative. Chapter 3 describes the development of a quantitative real-time PCR assay for the detection of BFDV DNA, using primers designed to amplify a conserved 81 bp fragment of open reading frame V1 (ORFV1) and SYTO9, a fluorescent intercalating dye. A synthetic oligonucleotide was used to make standard curves for the quantitation of viral load in blood and feather preparations.

PBFD is known to occur in a wide range of psittacine species, however there are no scientific or credible anecdotal reports of PBFD occurring in the cockatiel (*Nymphicus hollandicus*) despite it being one of the world’s most commonly kept companion bird species. Consequently this has resulted in speculation that the species may have some innate resistance to BFDV infection. Chapter 4 details an investigation into the presence and characteristics of BFDV in cockatiels using existing diagnostic tests (PCR, HA and HI assays).

In order to address the need for repeatable assays based upon consistent and standardised reagents, a recombinant BFDV capsid protein was produced and a specific monoclonal antibody developed against it. The behaviour of the protein in HA assays and the behaviour of the monoclonal antibody in western blotting, immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA) and HI assays is described in Chapter 5.
Chapter 5 also presents the methods used to develop a novel “blocking” (or “competitive”) ELISA (bELISA) for the detection of anti-BFDV antibodies in psittacine sera (Ab-bELISA). The assay was developed using a baculovirus-expressed recombinant BFDV capsid protein and a newly developed monoclonal antibody raised against this protein. The assay was then validated using 166 samples from eastern long-billed corellas (Cacatua tenuiostris) and 82 samples from the cockatiel serosurvey described in Chapter 4. Some of these birds were vaccinated with the recombinant capsid protein and some were non-vaccinated control birds, then all birds were challenged with live virus.

The successful control of PBFD in both pet and wild birds depends on the development of vaccines that incite a strong specific immune response and can be efficiently produced. Chapter 6 details the methods used to develop two DNA vaccines against BFDV, utilising the nucleotide sequence encoding a full-length and truncated capsid protein. The expression of the DNA vaccine constructs in vitro and in vivo was examined utilising an indirect immunocytochemistry assay and the Ab-bELISA described in Chapter 5. In addition, some options for the further development of these vaccines into effective methods for the control of BFDV are discussed.
Chapter 2. Review of the Literature

2.1 Introduction
Presented here is a review of the literature relevant to the research conducted for the development of this thesis. It is divided into 2 broad sections. The first section provides background and introductory information on the circoviruses. It discusses their classification, taxonomy, pathogenesis, and genomic structure and mode of replication. Particular attention is given to the mode of replication of circoviruses, based largely on studies conducted on porcine circovirus and homologous plant viruses (nano- and geminiviruses). The second section summarises the body of literature on beak and feather disease virus (BFDV), including its history, physical, chemical and molecular characteristics, pathophysiology, diagnosis, treatment and prevention.

2.2 The Circoviridae

2.2.1 Introduction and taxonomy
The system of nomenclature and classification used in this thesis is based on that proposed by Pringle (1999), Fauquet and Mayo (2001) and Fauquet et al. (2005). Genes and open reading frames (ORFs) are written in lowercase italics. Gene products (mRNA and proteins) are non-italicised and first letter is in uppercase. The definitions of “strain”, “serotype” and “isolate” are taken from Blood and Studdert (2000). A strain is defined as “one or more organisms within a species or variety, characterised by some particular quality”. A serotype is defined as “the type of a microorganism determined by its constituent antigens based on one of several different antibody-antigen reactions, or a taxonomic subdivision based thereon”. An isolate is defined as
“a population of microorganisms that has been obtained in pure culture from a field case or location”.

The Circoviridae are small, nonenveloped icosahedral animal viruses characterised by circular single-stranded DNA (ssDNA) genomes (Crowther et al., 2003). Viruses within the Circoviridae are further grouped into 3 genera. The genus Gyrovirus, of which chicken anaemia virus (CAV) is the only member, the genus Circovirus, which includes porcine circovirus 1 and 2 (PCV1 and PCV 2) and BFDV and the genus Anellovirus, comprising the human and animal Torque teno viruses (TTV) and human TT-like mini virus (TLMV) (Biagini, 2004) (Tables 2.1 and 2.2). The type species of Gyrovirus, Circovirus and Anellovirus is CAV, PCV1 and TTV, respectively. The phylogenetic relationship between CAV, PCV and BFDV is demonstrated in Figure 2.1.

Virus isolates which share features common to the Circoviridae but have not yet been formally classified by the ICTV are known as tentative circoviruses and anelloviruses. These viruses and their characteristics are summarised in Table 2.2. Because these viruses have been isolated from such a wide range of host species, it is likely that the Circoviridae are common within vertebrates. Certainly, since the majority of tentative members of the Circoviridae have been isolated from avian hosts, circoviruses are probably ubiquitous within the aves.

Related to the Circoviridae are the Nanoviridae, formerly known as the “plant circoviruses” and the Geminiviridae. Representatives of the Geminiviridae
include tomato yellow leaf curl virus (TYLCV) (Wartig et al., 1997), maize streak virus (MSV) (Boulton et al., 1989) and beet curly top virus (BCTV) (Briddon et al., 1989). Representatives of the Nanoviridae include subterranean clover stunt virus (SCSV) (Boevink et al., 1995), banana bunchy top virus (BBTV) (Wu et al., 1994), coconut foliar decay virus (CFDV) (Rhode et al., 1990) and faba bean necrotic yellow virus (FBNYV) (Katul et al., 1995). In fact it is hypothesised that circoviruses was the result of a recombination event between a nanovirus and a picorna-like virus such as a calicivirus (Gibbs and Weiller, 1999).

2.2.2 The viruses – history, clinical significance and biological properties

2.2.2.1 Genus Gyrovirus: chicken anaemia virus (CAV)

Chicken anaemia virus (CAV), first isolated in Japan in 1979 (Yuasa et al., 1979), causes disease in chickens less than 3 weeks old and in older, immunosuppressed birds. Mortality is usually around 10% (Cardona et al., 2000). Clinical disease is characterised by a severe anaemia, generalised atrophy of the lymphoid organs (bone marrow, spleen, thymus and bursa of Fabricus) and subcutaneous and intramuscular haemorrhages. The amount of virus detectable in the thymus and in clotted blood, which probably reflects replication in the bone marrow and viraemia, directly correlates with the severity of clinical signs (Tan and Tannock, 2005). All isolates characterised thus far belong to a single serotype (Von Bulow and Schat, 1997) and all known strains cause a disease syndrome known as chicken infectious anaemia (CIA) (Cardona et al., 2000). The genetic and ultrastructural characteristics are summarised in Table 2.1 and the phylogenetic relationship
between CAV and other members of the *Circoviridae* is demonstrated in Figure 2.1. The three-dimensional structure of CAV particles is an icosahedral T=1 structure arranged in 12 pentagonal, trumpet-shaped capsomers. The entire structure is made up of 60 capsid protein molecules (Crowther et al., 2003).

CAV can only be propagated in the T-cell lymphoblastoid cell lines MDCC-MSB1 (MSB1) and MDCC-JP2 and the B-cell line LSCC-1104B1 (Todd et al., 2001a), of which the MSB1 cell line is the most commonly used. Not all sub-lines of MSB1 cells are susceptible to infection, however and some sub-lines lose susceptibility following repeated subculture. In addition, some isolates of CAV will not grow or have reduced replication rates in particular MSB1 cell lines which are susceptible to other isolates (Lucio et al., 1990, Nogueira et al., 2007, Soine et al., 1994). This variation in cell tropism has been mapped to a hypervariable region within the protein VP1 (Renshaw et al., 1996). CAV infection of cultured cells causes a cytopathic effect (CPE), characterised by the death of some cells, enlarged swollen cells, alkaline medium and an inability to subculture (Todd et al., 2001a).

CAV usually grows to moderate ($10^5$ to $10^6$ TCID$_{50}$/0.1 mL) titres in MSB1 cells within 2-3 days of inoculation (Yuasa, 1983). After repeated passage, the pathogenicity of some strains is unchanged, whereas others become less pathogenic, however the reasons for these differences are unclear (Tan and Tannock, 2005, Todd et al., 1998). Depending on their location, mutations in the protein VP2 may either retain the ability to replicate but reduce *in vivo* pathogenicity (Peters et al., 2007), or may reduce replication,
cytopathogenicity and MHC1 down-regulation (Peters et al., 2006). Purified CAV is resistant to pasteurisation and after freeze drying is resistant to inactivation by dry heat up to 120ºC (Welch et al., 2006). The protein encoded by ORF3, apoptin, a protein that induces apoptosis via G2/M cell cycle arrest in transformed cells by interaction with the APC1 subunit of the anaphase promoting complex/cyclosome (APC/C) (Lee et al., 2007, Teodoro et al., 2004). Nucleocytoplasmic shuttling is required for proper induction of apoptosis (Heilman et al., 2006).

CAV is vertically transmitted in utero and via infected semen (Cardona et al., 2000, Hoop, 1992, Hoop, 1993) and can also be transmitted horizontally (Smyth et al., 2006a). The resulting immunosuppression is caused by destruction of T-lymphocyte precursors in the bone marrow and thymic cortex, as well as mature T lymphocytes in the spleen (Adair, 2000, Todd, 2000, Todd et al., 2001a). The virus may persist in the gonads of immune birds and can even be transferred to the embryos of chickens despite high virus neutralising (VN) antibody titres. It is unknown whether transfer is by infectious viral particles or as infective DNA, or whether this type of vertical infection is capable of inducing disease in infected chicks (Brentano et al., 2005), as maternal antibodies are considered to be protective against clinical disease (McNulty, 1991). Horizontal infections in birds older than 21 days of age, after maternal antibodies have waned, usually result in seroconversion but can also result in thymic lymphocyte depletion without anaemia or bone marrow atrophy (Smyth et al., 2006a).
Few studies have investigated antigenic variation between CAV isolates. Wang et al. (2007a) identified three epitopes on VP2, the third of which is immunodominant. Further research is required to determine whether these epitope sequences are involved in the scaffolding function of VP2.

2.2.2.2 Genus Circovirus: porcine circovirus 1 and 2 (PCV1 and PCV2) and beak and feather disease virus (BFDV)

Porcine circovirus – Porcine circovirus was the first animal circovirus to be discovered. Tischer et al. (1974) described “picornavirus-like particles” in the continuous pig kidney cell line PK-15 (ATCC-CCL 33). Experimental infection of pigs with the PK-15-derived PCV showed the virus to be non-pathogenic (Allan et al., 1995). However a new PCV, different to the PK-15 contaminant, was discovered associated with post-weaning multisystemic wasting syndrome (PMWS) (Clark, 1997). The two PCVs are distinct in terms of their antigenicity and DNA sequence (Table 2.1 and Figure 2.1) and the PK-15 contaminant and PMWS-associated virus have been designated PCV1 and PCV2, respectively. Like CAV, the structure of PCV has been demonstrated to be an icosahedral T=1 structure, made up of 60 capsid protein molecules arranged in 12 pentamers (Crowther et al., 2003). In contrast to CAV, however, PCV capsids have relatively flat capsomers.

PCV1 and PCV2 are most commonly grown in PK-15 cells. However they can grow and produce infectious viral particles in other cells of porcine origin and Vero, African green monkey kidney, cells (Allan et al., 1994a, Dulac and Afshar, 1989, Edwards and Sands, 1994, Misinzo et al., 2008b). PCV2 can replicate in macrophages, even though they are fully differentiated. Possibly
this occurs because the virus takes advantage of the upregulation of DNA polymerases in these cells after DNA damage (Meerts et al., 2005a). When grown in PK-15 cells, neither PCV1 nor PCV2 causes CPE under normal growth conditions. PCV1 has also been shown to infect bovine and ovine cells in vitro, but virus antigen was only observed in the cytoplasm of cells and no infectious virus was produced (Allan et al., 1994b). Tischer et al. (1995) demonstrated that PCV1 infected cell lines can be established by cloning persistently infected primary pig kidney cells and that these cell lines demonstrated properties consistent with transformation (immortalisation and loss of contact inhibition). As the passage number of these cells increased, however, the growth rate decreased and CPE was observed, suggesting that the transformed state was unstable. Cap is usually the first protein detectable in infected cells and the full replication cycle of PCV2 is between 24-36h (Meerts et al., 2005a).

Inhibition of endosome-lysosome system acidification reduces PCV2 infection of monocyte/macrophage cells, but enhances infection of epithelial cells in vitro (Misinzo et al., 2008b, Misinzo et al., 2005). Administration of gamma interferon (IFN-γ) to PK-15 cells and monocytic cells enhances replication of PCV2, but not PCV1 in vitro by increasing internalisation of virus-like particles (VLPs). Administration of alpha interferon (IFN-α) after inoculation also enhances infection, but pre-administration of IFN-α decreases infection in vitro. In PK-15 cells, combined administration of IFN-γ and inhibitors of endosome-lysosome system acidification increases the yield of PCV2 up to 50 times (Misinzo et al., 2008a). The use of ionophores, which inhibit endosome-lysosome system acidification, may even enhance PCV infection in
vivo, but this is yet to be confirmed experimentally. Purified PCV2 is resistant to pasteurisation and after freeze drying is resistant to inactivation by dry heat up to 120ºC (Welch et al., 2006) and in cell culture is resistant to heating at 75ºC for 15 minutes but inactivated after 15 minutes at 80ºC (O’Dea et al., 2008).

PCV1 fails to produce clinical disease in pigs after experimental infection and does not cause detectable cytopathic effects in cell culture (Allan et al., 1995, Tischer et al., 1982). PCV2 is the crucial pathogen in PMWS, a syndrome characterised by weight loss, fever, lymphadenopathy, dyspnoea and depletion of lymphocytes in the lymph nodes (Mankertz et al., 2004). PCV2 has also been associated with reproductive failure (West et al., 1999) and myocardial disease (Opriessnig et al., 2006b) and has been isolated from pigs with porcine dermatitis and nephropathy syndrome (PDNS) (Allan et al., 2000b). PCV2 is suspected to be immunosuppressive, as pigs infected with PCV2 are more susceptible to secondary infections with organisms such as Pneumocystis sp. or Chlamydophila spp. (Todd et al., 2001a) and infection prior to vaccination decreases the efficacy of vaccines (Opriessnig et al., 2006c).

Following natural PCV2 infection, IgM antibodies are detectable at around week 8 post-infection, peaking by 12 weeks and IgG antibodies are detectable at around week 10 post-infection, peaking by week 16 (Carasova et al., 2007). After experimental infection, IgM and IgG are detectable around 1 and 3 weeks post infection, respectively and peak IgM and IgG levels occur around 3 and 7 weeks post infection, respectively (Darwich et al., 2008). Viral load
has been reported to peak around week 10 following natural infection and persists into adulthood in piglets that survive (Carasova et al., 2007), whereas viral load is reported to peak around 3 weeks after experimental infection (Darwich et al., 2008). PCV2 actively replicates in most tissues (peripheral blood mononuclear cells (PBMCs), lymphoid tissues, lungs, liver, kidneys and spleen) by 14 days post infection, but PBMCs are only a transient site of replication (Yu et al., 2007). Replication begins in lymph nodes closest to the site of inoculation then spreads systemically. In early infections, viral replication in B lymphocytes is greater than in mononuclear cells and levels of viral DNA are consistent in monocytes, indicating that B lymphocytes are an important site of replication and that monocytes may be a site of viral persistence.

The development of an appropriate adaptive immune response is required for protective immunity to PCV2 (Meerts et al., 2005c). Levels of virus vary inversely with the amount of specific neutralising antibodies and IFN-γ present. Variations in the pattern of infection and immunity can be observed after inoculation of a group of pigs with the same strain of virus, indicating that the disease course is multifactorial (Meerts et al., 2005c) and probably depends on differences in individual host-virus interaction and the presence of other diseases or immune stimulation (Allan et al., 2000a, Allan et al., 2000c, Krakowka et al., 2001, Kyriakis et al., 2002, Meerts et al., 2005c, Opriessnig et al., 2006a, Opriessnig et al., 2003). Experimental inoculation of pigs with different strains of PCV2 has also been shown to induce different disease courses (Opriessnig et al., 2006d). Interestingly, the less pathogenic strain replicated to greater levels early in the course of infection but the more
pathogenic strain reached greater levels later and both isolates were genetically identical to the respective inocula when they were recovered from the experimentally infected pigs. PCV2 may be transmitted vertically (McIntosh et al., 2006), even if boars have circulating antibodies present (Larochelle et al., 2000). Serological surveys have shown that infection with PCV1 and PCV2 is widespread and likely occurs worldwide (Allan, 1996, Allan et al., 1998).

Further research, profiling serum cytokine expression, cytokine mRNA levels in lymphoid tissues and PBMCs in cell culture, suggests a T-cell mediated immunosuppression. Darwich et al. (2002) demonstrated that PCV2-positive pigs have lower levels of CD8\(^+\) and CD4\(^+\)/CD8\(^+\) PBMCs than negative pigs and this depletion in circulating lymphocytes correlates with a decreased level of circulating IgM\(^+\) lymphocytes. Furthermore, the amount of PCV2 genome in various lymphoid organs correlates with the degree of lymphocyte depletion in those organs. Stevenson et al. (2006) showed that levels of serum interleukin-10 (IL-10) and C-reactive protein are increased during experimental infection. Darwich et al. (2003b) demonstrated increased IL-10 mRNA in thymus tissues and IFN-\(\gamma\) mRNA in tonsils. Expression of mRNA of IL-2 and IL-12p40 in the spleen, IL-4 in tonsils and IFN-\(\gamma\), IL-10, IL-12p40 and IL-4 in inguinal lymph nodes was decreased. Overexpression of IL-10 was also histologically associated with thymic depletion. Darwich et al. (2008) determined that peak IL-10 levels in the PBMCs of experimentally inoculated pigs occurred at around 3 weeks post infection, at the same time as the peak in viral load and the inversion of the IgM/IgG ratio. PBMCs infected with PCV2 in cell culture responded by expressing IL-10 and IFN-\(\gamma\) and were less able to produce IL-4,
IL-2 or IFN-γ in response to challenge with mitogen or superantigen (Darwich et al., 2003a). The cultured PBMCs of experimentally inoculated pigs were also unable to produce IFN-γ after stimulation (Darwich et al., 2008).

PCV2 also reduces immune memory, via an IL-10 mediated downregulation of IFN-γ and IL-12 and an IL-10 independent downregulation of IL-2 (Kekarainen et al., 2008). In contrast to the upregulation of IFN-α in the PBMCs of previously naïve animals IFN-α production is also reduced in recall responses (Kekarainen et al., 2008). These findings are significant, as IL-10 has been suggested as the cause of immunosuppression leading to the persistence of other viruses (Brooks et al., 2006). Importantly, induction of IL-10 by PCV2 was independent of viral infectivity, suggesting that viral DNA is important in the regulation of host responses (Kekarainen et al., 2008, Vincent et al., 2007) and that interactions of viral and host proteins are probably not disturbed if infectivity is lost.

The ORFC2 protein of PCV2 has been shown to be involved in the immunopathogenesis of the virus. The protein causes apoptosis (Liu et al., 2005) via upregulation of the tumour suppressor gene p53 in a similar manner to many other viruses (Liu et al., 2007b). The ORFC2 protein of PCV2 also causes lymphoid depletion and reductions in CD4+ and CD8+ T-cells in experimentally infected mice (Liu et al., 2006b). However, Darwich et al. (2008) reported no significant differences in the relative proportions of CD4+ and CD8+ PBMCs by 69 days post infection in subclinical experimentally infected pigs. The PCV2 DNA genome has also been shown to have both inhibitory and stimulatory effects on the induction of alpha interferon (IFN-α)
and tumour necrosis factor – alpha (TNF-α) in natural interferon producing cells (NIPCs) (Hasslun et al., 2003, Stevenson et al., 2006, Wikstrom et al., 2007). The actions of the various oligodeoxyribonucleotides (ODNs) are independent of viral replication or the presence of the capsid protein and separate to the effect induced by non-PCV derived CpG ODNs. However, the secondary structure of the ODNs is important, as is the presence of a central CpG dinucleotide for IFN-α inducing ODNs, but not for inhibitory ODNs.

Various differences in antigenic composition, nucleotide sequences, replication kinetics and pathogenicity have been documented between PCV1 and 2 (Hughes and Piontkivska, 2007, Lefebvre et al., 2008, Mahe et al., 2000, Meehan et al., 1998) and between strains of PCV2 (Lefebvre et al., 2008, Meerts et al., 2005a, Opriessnig et al., 2006d), but specific changes accounting for the difference in pathogenicity between PCV1 and 2 or between PCV2 strains has not been documented. Lefebvre et al. (2008) found antigenic differences between PCV2 strains using a panel of monoclonal antibodies and Opriessnig et al. (2006d) found differences in virulence between PCV2 strains, but no specific differences have been found to account for differences in pathogenicity.

Five overlapping epitopes on PCV2 Cap (Lekcharoensuk et al., 2004) and conserved immunoreactive epitopes recognised by both B and T lymphocytes (Mahe et al., 2000, Stevenson et al., 2007) have been identified. Interestingly, whilst T lymphocyte responses to epitopes of PCV2 Cap were greater than reactions to epitopes of Rep or the protein encoded by ORFC2, only epitopes within Rep and the protein encoded by ORFC2 were immunodominant. This
indicates that T lymphocyte responses to PCV2 infection are primarily
directed against the non-structural proteins, or that T lymphocyte reactions to
Cap epitopes may depend on the conformation of those epitopes. Fan et al.
(2008) also proposed the presence of an immunodominant epitope in the N-
terminal of PCV2 Cap between amino acids 20-41, within the NLS, but this is
yet to be confirmed. Mahe et al. (2000) also identified epitopes within Rep that
cross-reacted serologically between PCV1 and 2. The proposed existence of
strains of PCV2 is supported by findings that different isolates cause different
disease courses (Opriessnig et al., 2006d) and display differences in patterns
of replication in vitro (Meerts et al., 2005a).

*Beak and feather disease virus* – A summary of the genetic and
ultrastructural characteristics is presented in Table 2.1 and a detailed
description of BFDV is provided in Section 2.3, below.

*Other circoviruses* – Pigeon, goose and canary circovirus have all been
officially classified as members of the genus *Circovirus* (Fauquet et al., 2005)
and they share similar genomic and structural characteristics to other
circoviruses (Table 2.1) and are more closely related to BFDV than PCV or
CAV (Figure 2.1). Of these, PiCV is the best studied and is associated with
lethargy, anorexia, runting and poor racing performance as part of a
multifactorial syndrome known as young pigeon disease syndrome (YPDS)
(Raue et al., 2005). PiCV is distributed worldwide (Coletti et al., 2000,
Hattermann et al., 2002, Soike et al., 2001) and in-situ hybridisation, a dot-blot
assay and standard and multiplexed PCR assays have been developed to
detect viral DNA (Freick et al., 2008, Smyth et al., 2001, Todd et al., 2006, Todd et al., 2002).

Goose circovirus is associated with a runting syndrome (Soike et al., 1999) and is also expected to have a worldwide distribution (Ball et al., 2004, Chen et al., 2003). Dot-blot and PCR assays have been developed for the detection of viral DNA (Ball et al., 2004) and an indirect immunofluorescence assay using a recombinant GoCV capsid protein has been developed for serological screening (Scott et al., 2006). CaCV was isolated from systemically ill canaries (*Serinus canaria*) and its genome has been sequenced (Phenix et al., 2001).

### 2.2.2.3 Genus *Anellovirus*: torque teno viruses (TTVs) and TT-like mini virus (TLMV)

Torque teno virus (TTV) was first discovered in the plasma of a Japanese patient (initials T. T.) with post-transfusion non-A-G hepatitis (Nishizawa et al., 1997) and TT-like mini virus (TLMV) discovered as a result of a survey investigating the prevalence of TTV (Takahashi et al., 2000b). The genetic and ultrastructural characteristics are summarised in Table 2.1. Like other members of the *Circoviridae*, TTV has an icosahedral structure (Itoh et al., 2000). TTV may be cultured *in vitro* using the Chang Liver cell line, the Raji B lymphoblast cell line or PBMC cultures stimulated with phytohaemagglutinin (PHA), lipopolysaccharide (LPS) and IL-2 (Desai et al., 2005, Mariscal et al., 2002).
Despite numerous studies, TTV has yet to be definitively associated with a given lesion, thus it is considered an “orphan” virus (Bendinelli et al., 2001). TTV is widespread amongst the general population and has a worldwide distribution (Hohne et al., 1998, Niel et al., 1999, Prescott and Simmonds, 1998, Takahashi et al., 1998). Viral DNA can be detected in sera, semen, saliva, stool, breast milk, vaginal secretions and cord blood, suggesting that the virus may be transmitted both vertically and horizontally (Deng et al., 2000, Fornai et al., 2001, Inami et al., 2000, Jones et al., 2005, Komatsu et al., 2004, Lin et al., 2000, Matsubara et al., 2001, Matsubara et al., 2000, Ohto et al., 2002, Ross et al., 1999, Saback et al., 1999). TTV infections are long lasting and possibly permanent (Bendinelli, 2001, Bendinelli et al., 2001, Maggi et al., 2001), but clearance of the virus after treatment with IFN-α has been reported (Akahane et al., 1999, Hagiwara et al., 1999). TTV is also considered to be extremely resistant and may persist in the environment for long periods of time (Haramoto et al., 2005).

TT viruses have also been discovered in non-human species including: chimpanzees, pigs (Sd-TTV), dogs (Cf-TTV), cats (Fc-TTV), sheep, cattle, chickens and tupaia (tree shrews) (Tbc-TTV) (Cong et al., 2000, Leary et al., 1999b, McKeown et al., 2004, Okamoto et al., 2001, Verschoor et al., 1999). Swine TTV has a worldwide distribution (Brassard et al., 2008, Martelli et al., 2006, McKeown et al., 2004) and is ubiquitous in both domestic and wild pigs (Martinez et al., 2006). Swine TTV DNA can be detected in up to 90.5% of plasma samples and 60.3% of faecal samples (Brassard et al., 2008) and can also be detected in boar semen, implying that the virus may be transmitted vertically (Kekarainen et al., 2007). Bovine TTV has been detected in 1.1% of
cattle in Quebec (Brassard et al., 2008), but worldwide studies are limited. Even though Sd-TTV has not been shown to be pathogenic, higher levels of Sd-TTV DNA are detectable in pigs with PMWS, though this likely reflects the effect of PCV2 rather than Sd-TTV. As yet there is no evidence that animal TTVs can be transmitted to humans and TTVs have been shown to have poor viability in heterologous species (Mushahwar et al., 1999). Still, human TTV has been transmitted to chimpanzees and macaques (Takahashi et al., 2000a, Tawara et al., 2000) and so the significance of animal TTVs in terms of food safety and xenotransplantation requires further investigation.

Figure 2.1: Phylogenetic relationship between chicken anaemia virus (CAV), porcine circovirus 1 and 2 (PCV1 and 2), beak and feather disease virus (BFDV) and some other avian circoviruses [pigeon circovirus (PiCV), goose circovirus (GoCV), canary circovirus (CaCV) and duck circovirus (DuCV)]. Neighbour-joining analysis with 1000 bootstrap cycles. Adapted from Hattermann (2003), numbers at the nodes indicate bootstrap support, bar indicates distance.
2.2.3 Genome organisation

The genome size, described gene products, predicted ORFs and putative gene products of CAV, PCV1 and BFDV, as well as the anelloviruses (TTVs and TLMV) and some novel circoviruses, are displayed in Table 2.1. The genome organisation of these viruses is depicted in Figure 2.2. Where possible, the ORF-labelling used conventions for geminiviruses (Meehan et al., 1997, Niagro et al., 1998) are also used here.

The genomes of viruses within the genus *Circovirus* are organised in a similar manner, but are different to those of CAV, the only member of the genus *Gyrovirus* and members of the genus *Anellovirus*. CAV is unique within the animal DNA viruses in that it uses an unspliced, polycistrionic message for synthesis of its proteins (Todd et al., 2001a). The CAV genome contains 3 partially overlapping proteins and transcriptional analysis has demonstrated that one major transcript, of approximately 2.1 kb is produced and encodes all 3 ORFs (Noteborn et al., 1992). Further to this, the transcript was found to be complementary to the encapsidated DNA and its genome can thus be characterised as negative-sense (Noteborn et al., 1992). Kamada et al. (2006) also found transcripts of 1.6, 1.3 and 1.2 kb, but their precise significance is unknown. ORF1 encodes VP1, the capsid protein (Noteborn et al., 1992), which also has elements of replicase-associated proteins. ORF2 encodes VP2, a dual-specificity protein phosphatase (Peters et al., 2002, Peters et al., 2005) and ORF3 encodes apoptin. Both VP1 and VP2 are required for the proper formation of virions (Lacorte et al., 2007, Noteborn et al., 1998), so VP2 likely acts as a scaffolding protein. Furthermore, both VP1 and VP2 are required for the development of protective immunity (Noteborn et al., 1998).
Mutational analysis of apoptin has shown that it has an N-terminal nuclear export signal (NES) and a C-terminal nuclear localisation signal (NLS) and that multimerisation of the protein is required for the NES to be functional (Heilman et al., 2006).

The members of the genus *Circovirus* differ from CAV in that they have ambisense genomes; i.e. they require proteins encoded by both the viral and complementary strands of the genome in order to replicate (Niagro et al., 1998).

Within the genomes of the porcine circoviruses, 4 ORFs have been consistently identified in PCV2 sequences and up to 7 ORFs have been identified within PCV1. The whole-sequence nucleotide identity within PCV2 isolates has been shown to be >96%, but <80% identity between PCV1 and PCV2 isolates (Meehan et al., 1998). Transcriptional analysis and studies using infectious clones of PCV genomes have demonstrated 3 mRNA species, of 1230+/−50, 990+/−50 and 750+/−50 nt in size (Mankertz et al., 1998) as well as an NLS located at the N-terminal of the capsid protein and the replication-associated proteins (Finsterbusch et al., 2005, Liu et al., 2001) and IFN-α modulatory CpG motifs (Haslfgang, 2003). The nucleotides between the V1 and C1 ORFs also contain a putative stem-loop structure with a nonanucleotide motif that is thought to play a key role in rolling circle replication.

Both PCV1 and 2 have three ORFs in common: ORFC1, V1 and C2. The C1 ORF encodes the capsid protein (Cap), the major antigenic determinant
(Blanchard et al., 2003, Mahe et al., 2000, Nawagitgul et al., 2000, Zhou et al., 2005) and the V1 ORF encodes the two replication-associated proteins (Rep and Rep’) (Finsterbusch et al., 2005, Mankertz and Hillenbrand, 2001, Mankertz et al., 2003, Meehan et al., 1997). Rep’ is generated by mRNA splicing, as occurs with geminiviruses (Schalk et al., 1989). Four amino acid sequences required for replication, conserved across nano- and geminiviruses, are also found in PCV1 Rep. The first three sequences are found at the N-terminus of the protein and the fourth in the middle. The first conserved motif is FLTNN, the second is HLQGF, involved in metal ion binding (Ilyina and Koonin, 1992), the third, YCSK, is a catalytic site for DNA cleavage (Laufs et al., 1995a) and the fourth, GKS, is a dNTP binding site (Ilyina and Koonin, 1992). The promoter of the capsid gene (cap) has been mapped to nucleotides 431-507 (Mankertz and Hillenbrand, 2002). Liu et al. (2005, 2006) demonstrated that the ORFC2 protein, whilst not necessary for viral replication, plays an important role in the pathogenesis of the disease.

Similar genomic features to those described above have been reported for BFDV. Like PCV, the major ORFs and C1 and V1, which encode similar Cap and Rep proteins, respectively (Bassami et al., 1998). Similarly, the Cap protein of BFDV is also the major antigenic determinant (Stewart et al., 2007). Bassami et al. (1998) described the potential stem-loop structure and nonanucleotide motif between ORFs V1 and C1 and identified up to 7 ORFs that code for potential proteins of >8.7 kDa. In contrast, Niagro et al. (1998) identified only the larger ORFs: V1, C1 and C2 and Ritchie et al. (1989b) identified 3 proteins from virus purified from feather homogenates (Table 2.1). Transcriptional analysis, using RNA extracted from feather pulp,
demonstrated transcripts synthesised from both reading frame strands (Niagro et al., 1998). Studies using recombinant proteins have demonstrated the presence of an NLS (Heath et al., 2006) within BFDV Cap, similar to that of PCV. Similar amino acid motifs involved in rolling-circle replication initiation are present in BFDV Rep, as is a putative NES (unpublished observations). BFDV has been shown to cause apoptosis in the skin of infected birds (Trinkaus et al., 1998) and the ORFC2 protein, which is similar to that of PCV2, may be responsible for this.

Figure 2.2: Genome organisation of A: Chicken Anaemia Virus, B: Porcine Circovirus 2, C: Beak and Feather Disease Virus, D: Torque-teno Virus and E: TT-like Mini Virus showing putative open reading frames on the viral (V) and capsid (C) strands. Adapted from Todd (2001a) and Okamoto et al. (2001)

The human and animal TTVs and TLMV share similar genomic features (Tables 2.1 and 2.2) and are more similar to CAV than PCV or BFDV, as they have negative-sense genomes. The genomes of human, chimpanzee, tupaia, swine, dog and cat TTVs have been characterised (Okamoto et al., 2001,
Okamoto et al., 2002, Verschoor et al., 1999). Genome size varies markedly within the genus *Anellovirus*, the largest of which described so far is human TTV at 3.8 kb and the smallest feline TTV at 2.1 kb. The genome of the anelloviruses is separated into a coding region and a non-coding region. Two partially overlapping ORFS can be deduced directly from the sequence of the coding region, the largest of which encodes a protein capsid homologous to the capsid protein of CAV and contains elements characteristic of the Rep proteins of the circoviruses (Biagini et al., 2001). The function of the protein encoded by ORF2 is unknown, but it contains sequences characteristic of tyrosine phosphatases (Biagini et al., 2001).

Transcriptional analysis has revealed 3 mRNAs from both the human and swine TTV genomes. The mRNAs from human TTV are 2.9, 1.2 and 1 kb long, revealing the existence of ORF3 which is obtained by splicing (Okamoto et al., 2000). The non-coding region occupies between 24-31% of the genome, depending on the animal it was isolated from (Leary et al., 1999a, Miyata et al., 1999, Okamoto et al., 2002). It contains promoter and enhancer elements (Kamada et al., 2004), a GC-rich sequence that probably forms stem-loop structures (Okamoto et al., 2002) and regulatory sequences involved in rolling-circle replication (Mankertz et al., 2004).

Due to the amount of genetic variation between TTV isolates, 5 distinct genogroups exist within human TTV isolates (Devalle and Niel, 2004, Peng et al., 2002) and 2 genogroups exist within Sd-TTV isolates (Niel et al., 2005). Infection with multiple genogroups is common (Bendinelli et al., 2001, Jelcic et al., 2004, Khudyakov et al., 2000, Niel et al., 2005) and each genogroup
produces comparable viral loads single-genogroup infections. However, a form of infection facilitation may exist between genogroups 1 and 3, as co-infection with these genogroups produces higher viral loads than would ordinarily be expected (Maggi et al., 2005).

The Circoviridae are genetically diverse, even within species. A consistent pattern, though, is that the synonymous nucleotide diversity of rep is always lower than cap of circoviruses, indicating functional constraint on the gene (Hughes and Piontkivska, 2007). Evidence of recombination has been found in both BFDV (Heath et al., 2004) and PCV2 (Ma et al., 2007, Olvera et al., 2007), as well as CAV (He et al., 2007), human TTV and TLMV (Biagini et al., 2001, Worobey, 2000) and the viruses are subject to past and ongoing purifying selection (Hughes and Piontkivska, 2007, Olvera et al., 2007).

Hughes and Piontkivska (2007) also found that PCV2 rep showed a strong excess of rare polymorphisms, supporting theories of a population bottleneck followed by rapid worldwide spread of the virus (Meehan et al., 1998). Nei (2007) postulated that differences in pathogenicity may also arise as a result of effectively neutral mutations, as opposed to selection by Darwinian means. The nucleotide analysis conducted by Hughes and Piontkivska (2007) seems to support that this has occurred in the case of PCV2. As yet, no association has been found between the genotype and pathotype of any of the members of the Circoviridae. However, given the effect of random mutations, recombination and purifying selection this may occur, but will require experimental confirmation.
### A. Circoviridae

<table>
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<tr>
<th>Genus</th>
<th>Virus</th>
<th>Host Species</th>
<th>Size (nm)</th>
<th>Density (g/mL)</th>
<th>Genome Size (kb)</th>
<th>No. of ORFs</th>
<th>Putative gene products (kDa)</th>
<th>Described gene products (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCV2</td>
<td>Pig (Suis domesticus)</td>
<td>20.5</td>
<td>1.7-1.8</td>
<td></td>
<td>V1: 314 aa, nt 51-995 C1: 233 aa, nt 1725-1034 C2: 104 aa, nt 671-357 C3: 59 aa, nt 565-386</td>
<td>Capsid: 26.3 Rep: 23.7 Other: 15.9</td>
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<tr>
<td>TTV</td>
<td>15, 16, 23-24</td>
<td>Human (Homo sapiens)</td>
<td>30-32</td>
<td>1.31-1.35</td>
<td>3.8</td>
<td>ORF1: 760-770 aa ORF2: 120 aa, nt 1613-154 ORFC1: 250 aa, nt 1761-1009</td>
<td>ORF1 (Rep): 33.4 ORFC1 (Capsid): 30 ORFC2: 14.9</td>
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### B. Nanoviridae

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<th>Density (g/mL)</th>
<th>Genome Size (kb)</th>
<th>No. of ORFs</th>
<th>Putative gene products (kDa)</th>
<th>Described gene products (kDa)</th>
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<td>Anellovirus</td>
<td>TTV</td>
<td>Human (Homo sapiens)</td>
<td>&lt;30</td>
<td>1.31-1.34</td>
<td>2.9</td>
<td>ORF1: 675 aa, nt 529-2556 ORF2: 100 aa, nt 341-840</td>
<td>Capsid: 19</td>
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</tbody>
</table>

References: 1(Todd et al., 1990), 2(Todd et al., 1991b), 3(Todd et al., 2001a), 4(Crowther et al., 2003), 5(Claessens et al., 1991), 6(Peters et al., 2002), 7(Niagro et al., 1998), 8(Tischer et al., 1982), 9(Mankertz et al., 2004), 10(Meehan et al., 1997), 11(Todd, 2004), 12(Bassami et al., 1998), 13(Bassami et al., 2001), 14(Ritchie et al., 1989b), 15(Biagini, 2004), 16(Biagini et al., 2001), 17(Takahashi et al., 2000b), 18(Todd et al., 2001c), 19(Coletti et al., 2000), 20(Phenix et al., 2001), 21(Todd et al., 2008), 22(Yu et al., 2007b), 23(Itoh et al., 2000), 24(Ninomiya et al., 2007)
Table 2.2: Comparison of the genomic organisation and gene products of some tentative members of the Circoviridae. A. novel circoviruses, B. novel anelloviruses

<table>
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<tr>
<th>A. Novel circoviruses</th>
<th>Virus</th>
<th>Host Species</th>
<th>Genome Size (kb)</th>
<th>No. of ORFs</th>
<th>Putative gene products (kDa)</th>
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<td>DuCV&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Duck (Anas sp.)</td>
<td>1.996</td>
<td>ORFV1: 292 aa, nt 48-927</td>
<td>ORFV1 (Rep): 33.6</td>
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<td>ORFV2: 88 aa, nt 897-1163</td>
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<td>ORFV3: 9.7</td>
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<td>ORFC3: 78 aa, nt 400-164</td>
<td>ORFC3: 9.2</td>
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<td>ORFV1 (Rep): 33.2</td>
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<td>ORFC1: 276 aa</td>
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<td>ORFC2: 106 aa</td>
<td>ORF3: 11</td>
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<td>RaCV&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Raven (Corvus coronoides)</td>
<td>1.898</td>
<td>ORFV1: 291 aa, nt 36-911</td>
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<td>ORF3: 224 aa, nt 430-647 and 2301-2484</td>
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<td>ORF3: 282 aa, nt 350-714 and 2323-2803</td>
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B. Novel anelloviruses

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<td>Tbc-TTV&lt;sup&gt;11&lt;/sup&gt;</td>
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<td>ORF3: 214 aa, nt 343-519 and 1455-1920</td>
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References: <sup>1</sup>(Hattermann et al., 2003), <sup>2</sup>(John et al., 2006a), <sup>3</sup>(Stewart et al., 2006), <sup>4</sup>(Todd et al., 2007), <sup>5</sup>(Halami et al., 2008), <sup>6</sup>(McKeown et al., 2004), <sup>7</sup>(Okamoto et al., 2002), <sup>8</sup>(Kekarainen and Segales, 2008), <sup>9</sup>(Verschoor et al., 1999), <sup>10</sup>(Cong et al., 2000), <sup>11</sup>(Takahashi et al., 2000b), <sup>12</sup>(Okamoto et al., 2001)
2.2.4 Mode of replication
The replication strategy of any DNA virus must follow certain predetermined steps: 1) entry of the virus into the cell and transportation to the nucleus, 2) import of the viral genome to the nucleus, 3) import of viral proteins, 4) replication of the genome and transcription of mRNAs, 5) export of mRNAs for translation in the cytosol, 6) import of viral proteins to the nucleus after translation and virion assembly, 7) export of assembled viral capsids from the nucleus, 8) exit of virions from the cell (adapted from Whittaker and Helenius, 1998). The Geminiviridae, Nanoviridae and Circoviridae utilise a number of methods to overcome these obstacles.

Entry and transport to the nucleus – PCV2 has been shown to use heparin sulphate and glycosaminoglycan receptors to attach to monocyte/macrophage cells (Misinzo et al., 2006), enters via clathrin-mediated endocytosis and is dependent on endosomal acidification for infection (Misinzo et al., 2005) (Figure 2.3a). The mechanism of entry into epithelial cells, however, is unknown. The NLS probably plays a role in viral attachment and entry, since protein binding to these attachment receptors is limited to the basic amino acids lysine and arginine (Esko, 1999) and the NLS of circoviruses (and many other viruses) has a high proportion of arginine residues. It is likely that BFDV uses a similar mechanism for cellular entry and activation, although this remains unknown. Uncoating of the viral genome occurs by serine protease-mediated capsid disassembly within endosomes (Misinzo et al., 2008b).

Once the virus has entered the cell, the viral DNA must then be transported to the nucleus. The coat protein of geminiviruses encodes an NLS (Kunik et al.,
1998, Palanichelvam et al., 1998, Qin et al., 1998, Soto et al., 2005) and studies by Finsterbusch et al. (2005) and Shuai et al. (2008) on PCV1, Liu et al. (2001) on PCV2 and Heath et al. (2006) on recombinant BFDV Cap have demonstrated that the capsid protein localises to the nucleus of cells and binds both single-stranded and double-stranded DNA (ssDNA and dsDNA, respectively). Heath et al. (2006) also demonstrated that co-expression of Rep and Cap localised Rep to the nucleus, but without Cap, Rep was located in the cytoplasm. Thus, Cap is likely to be responsible for the transport of the viral DNA and Rep into the nucleus of the cell for replication in a similar manner to that exhibited by geminiviruses (Qin et al., 1998). The Rep and Rep’ of porcine circoviruses, however, contain their own NLS at the N-terminus of the proteins and do not require the assistance of Cap to locate to the nucleus (Finsterbusch et al., 2005). Transport to the nucleus likely occurs by binding of Cap to dyneins and transport along microtubules (Radtke et al., 2006), but this has not yet been investigated in the case of circoviruses.

**Import of the viral genome to the nucleus** – No studies to date have examined the mode of entry of circoviruses into the nuclei of infected cells, however it is known that other DNA viruses are able to transfer their genome through the nuclear pore complex (NPC) (Corbett and Silver, 1997, Whittaker and Helenius, 1998). Nuclear localisation signals have been shown to interact with importin α5 and then importin β in the process of docking with NPCs and the sites of DNA binding and importin interaction are close together (Fagerlund et al., 2002). As such, the situation with circoviruses is probably as follows: after uncoating and binding the DNA genome, the NLS of the capsid protein docks with the NPC via interaction with importins and then DNA
translocation occurs via a cycle of GTP hydrolysis (Corbett and Silver, 1997) (Figure 2.3a). Alternately, the viral genome may enter the nucleus during mitosis by associating itself with cellular chromatin (Whittaker and Helenius, 1998).

**Import of viral proteins** – Import of circovirus proteins likely occurs by interaction of the NLS with NPCs and subsequent energy-dependent transfer (Figure 2.3a). Both the Cap and Rep proteins of the porcine circoviruses contain NLSs (Finsterbusch et al., 2005, Liu et al., 2001), as does BFDV Cap (Heath et al., 2006). In contrast to the replicase-associated proteins of the PCVs, BFDV Rep does not contain an NLS, instead interaction between Cap and Rep is required for nuclear localisation of Rep (Heath et al., 2006). Transfer of circoviral proteins probably occurs in their native form, since proteins up to 39nm in diameter may pass through the NPCs in this manner (Dworetzky and Feldherr, 1984, Pante and Kann, 2002).

**Genome replication and mRNA transcription** – Since circoviruses have small genomes and hence limited coding capacity, they are highly dependant on cellular enzymes for their replication. Indeed, they are the smallest viruses known to cause disease. The dependence on cellular functions for replication is supported by observations by Tischer et al. (1987) that PCV1 requires mitosis to be taken up into the nucleus and that CAV antigen can be detected in only 30-40% of cells in CAV-infected MSB1 cell cultures – this proportion probably corresponds to cells which have undergone division. Various studies suggest that replication not only requires the presence of cellular enzymes, but Cap and Rep as well (Bol, 2005, Boulton et al., 1989, De Graaff et al.,

Once the viral DNA is in the nucleus of the cell, replication can commence. Like circoviruses, bacteriophages and plant nano- and geminiviruses also have circular, ssDNA genomes and studies of their modes of replication (Hanley-Bowdoin et al., 1999) have been useful in understanding the replication of circoviruses. The first step in the replication cycle for bacteriophages, plant geminiviruses and circoviruses alike is the synthesis of a complementary strand to form the dsDNA replicative form (RF) (Figure 2.3a) and this is most likely performed using cellular enzymes (Gassmann et al., 1988). The first 16-20 amino acids of the arginine-rich N-terminal of the capsid protein may play a role in formation of the RF, in a similar manner to the last 19 amino acids of the C-terminus of Hepatitis B virus (HBV) (Nassal, 1992). With members of the genus circovirus, once this RF is produced, replication most likely continues by rolling circle replication (RCR) (Figure 2.3b), as occurs with bacteriophage φX174, the best known example of RCR. Geminivirus Rep proteins have been shown to contain conserved motifs similar to those used by bacterial plasmids for RCR (Koonin and Ilyina, 1992). Similar motifs have also been discovered in PCV2 (Mankertz and Hillenbrand, 2001) and exist in BFDV Rep (unpublished observations). The situation with CAV (genus Gyrovirus) is less clear and will be discussed below.

Replication of PCV (and probably BFDV) begins when the positively-charged endonuclease domain of Rep and Rep’ (Mankertz and Hillenbrand, 2001,
Vega-Rocha et al., 2007) (encoded by ORFV1) binds to the origin of replication (or minimal binding site (MBS)) (Steinfeldt et al., 2001, Steinfeldt et al., 2006) (Figure 2.3b), the 3’ part of the inverted repeat (5’-T/AAGTATTAC-3’) and the twice-repeated 8 bp motif (5’-GGGGCACC-3’), located between the stem-loop and the initiation codon of ORFV1 (Bassami et al., 1998, Mankertz et al., 2000, Niagro et al., 1998, Steinfeldt et al., 2001). It is important to note that both Rep and Rep’ are required by the porcine circoviruses for initiation of replication (Mankertz and Hillenbrand, 2001, Mankertz et al., 2003), so the transcription, mRNA export, translation and re-import of at least a few molecules of Rep and Rep’ are probably required before replication can begin.

Both Rep and Rep’ bind to the origin of replication (Mankertz and Hillenbrand, 2001, Mankertz et al., 2003) and both contain certain conserved motifs associated with RCR (Ilyina and Koonin, 1992). The first three sequences are found at the N-terminus of the protein and the fourth in the middle. The first conserved motif is FLTNN, the second is HLQGF, involved in metal ion binding (Ilyina and Koonin, 1992), the third, YCSK, is a catalytic site for DNA cleavage (Laufs et al., 1995a) and the fourth, GKS, is a dNTP binding site (or P-loop) (Ilyina and Koonin, 1992). Rep’ lacks this dNTP binding site, so it may function as a nickase, whereas Rep likely functions as a helicase (Mankertz et al., 2003). Interestingly, the replication-associated proteins of the porcine circoviruses are conserved to an extent that they are interchangeable (i.e. Rep and Rep’ of PCV1 efficiently initiate replication of PCV2 and vice versa) (Mankertz et al., 2003). As yet it is not known whether BFDV also utilises 2 transcripts of the replication-associated protein.
In the presence of divalent metal ions, the endonuclease activity of Rep (Mankertz and Hillenbrand, 2001, Vega-Rocha et al., 2007) then nicks the conserved nonanucleotide motif at the apex of the stem-loop structure (Hanley-Bowdoin et al., 1999, Laufs et al., 1995b, Steinfeldt et al., 2006) (Figure 2.3b). Mutation of the first four nucleotides (Steinfeldt et al., 2006) and mutations within the nonanucleotide motif of PCV (Mankertz et al., 1997) result in total loss of replicative function, as do mutations within the hexanucleotide motif (analogous to the octanucleotide of BFDV) (Cheung, 2006). The stem of the stem-loop then acts as a primer and the DNA is extended by cellular DNA polymerases and displaces the original virus strand (Gassmann et al., 1988, Rigden et al., 1996) (Figure 2.3b). Once the circle has been completed, Rep cleaves the displaced virus strand, then ligates the displaced strand to form another circular ssDNA viral strand (Laufs et al., 1995b, Steinfeldt et al., 2006) (Figure 2.3b).

The excision of unit-length viral genomes is accomplished primarily via this rolling-circle replication method; homologous recombination plays only a minor role (Cheung, 2007). The stem-loop structure is not necessary for the cleavage of the nonanucleotide motif, as Rep is able to cleave both single-stranded and double-stranded DNA, independent of the presence or absence of a hairpin within the sequence (Steinfeldt et al., 2006). However, the ligation of the newly synthesised DNA by Rep is dependant on the close proximity of the strands and this is accomplished by the hairpin structure of the stem-loop (Steinfeldt et al., 2006). Cap likely plays a role in the regulation of virus replication, as geminivirus Cap has been shown to downregulate the
replication initiation function of Rep, controlling the conversion of ssDNA into dsDNA RFs (Malik et al., 2005).

**Export of mRNAs for translation** – How circovirus mRNAs are exported from the nucleus for translation is not known, but export is likely to occur via the interaction of an NES in one of the viral proteins with the NPC (Whittaker and Helenius, 1998) (Figure 2.3a). BFDV Rep has been shown to localise to the cytoplasm (Heath et al., 2006) and may contain a putative NES (unpublished observations), but the situation with the PCVs is unclear, since both Rep and Rep' contain NLSs. Alternately, another as-yet undescribed protein with a nuclear shuttling function may be responsible.

**Import of viral proteins and virion assembly** – After translation, import of the viral proteins likely occurs by interaction of the NLS (Finsterbusch et al., 2005, Heath et al., 2006, Kunik et al., 1998, Liu et al., 2001) with NPCs, or interaction between Cap and Rep in the case of BFDV (Heath et al., 2006) (Figure 2.3a).

Packaging of the newly synthesised viral DNA into encapsidated virions likely requires the participation of Cap. Nassal (1992) found that removal of the 19 C-terminal amino acids of the C protein of HBV reduced the production of mature DNA genomes, but the genomes present were properly encapsidated. The N-terminus of the NLS of circoviral capsid proteins likely has a similar function, given the similar composition of the arginine-rich N-terminus of Cap.
**Export of assembled virions** – How assembled virions are transported into the nucleus to form the characteristic paracrystalline arrays is not precisely known, but interaction between the NES and NPC is the likely method (Figure 2.3a). CAV apoptin contains both an NLS and NES and multimerisation of apoptin is required for nuclear export of the protein (Heilman et al., 2006). Cap and Rep of BFDV are known to interact (Heath et al., 2006), so perhaps multimerisation of Rep facilitates transport of Cap into the cytoplasm. Alternately, circoviruses may use as-yet undescribed shuttling proteins produced in small amounts at the end of the replication cycle, in a similar manner to the geminiviruses (Qin et al., 1998). If nuclear export does occur using an NES, it likely occurs by interaction of the NES with CRM1 and export through the NPC (Henderson and Eleftheriou, 2000). Transport away from the nucleus likely occurs by binding of Cap to kinesins and transport along microtubules (Radtke et al., 2006), but this has yet to be specifically investigated in circoviruses.

**Export of virions from the cell** – Geminiviruses use a second specialised shuttle protein to transport virions out of the cell (Qin et al., 1998, Sanderfoot and Lazarowitz, 1995), but the means by which circoviruses exit the cell is unknown. Epithelial cells are a common site of replication and have a rapid rate of turnover (Ross et al., 1995), so a possibility is the release of virions when cells degrade as part of the normal cycle of cell replacement.

The mode of replication of CAV is not well understood. None of the CAV proteins described thus far share similarities with the replication-associated proteins of other animal circoviruses or plant geminiviruses. However, the
capsid protein does contain the 3 peptide motifs involved in RCR (Ilyina and Koonin, 1992) and the VP2 protein is required for efficient replication (Peters et al., 2006, Peters et al., 2002). The nonanucleotide motif found in other ssDNA viruses is semiconserved, but does not occur at the apex of a stem-loop motif, so it is unknown whether it represents the cleavage site. Niagro et al. (1998) suggested that replication of CAV likely involves host cell topoisomerase and/or helicase to provide an unwinding function. Multimerisation of apoptin is required for nuclear export and shuttling of apoptin is required for the induction of apoptosis (Heilman et al., 2006), so apoptin may play a role in the movement of other viral proteins and nucleic acids.
Figure 2.3: Example of proposed method of replication of circoviruses. A: Movement of viral components into various cellular compartments 1. entry of the virus to the cell and uncoating in endosomes, 2. transfer of viral DNA and cap to the nucleus via the nuclear pore complex (NPC), 3. generation of the double-stranded replicative form (RF), production of mRNAs and genome replication, 4. export of mRNAs to the cytoplasm for translation in the rough endoplasmic reticulum (RER), 5. transport of viral proteins to the nucleus through the NPC, 6. packaging of virions, export to the cytoplasm and formation of paracrystalline arrays. B: Rolling circle replication. 1. double-stranded DNA RF, 2. binding of Rep, 3. nicking of the stem-loop by Rep and binding of host cell polymerase (pol), 4. displacement of the original viral strand and ligation of the displaced strand, creating a new single-stranded DNA (ssDNA). The new ssDNA can then be encapsidated or form a new RF and continue rolling circle replication.
2.3 Beak and Feather Disease Virus

2.3.1 Introduction
A BFDV-like syndrome was first described in 1907, affecting red-rumped grass parakeets (*Psephotus haematonotus*). Affected birds were described as “quite healthy, except being destitute of feathers” (Ashby, 1907). This disease was attributed as being responsible for the decline of the species in the Adelaide Hills, as affected birds were likely to be more susceptible to predation.

Pass and Perry (1984) were the first to describe the disease in captive birds in detail; they also coined the term “psittacine beak and feather disease syndrome” and postulated a viral cause. McOrist et al. (1984) described a similar syndrome in wild sulphur-crested cockatoos (*Cacatua galerita*) around the same time. The condition in budgerigars, known as “French moult”, had been recognised by aviculturists since the early 1970s. It has since come to be recognised as the most common disease of wild and captive psittacines, with a worldwide distribution (Albertyn et al., 2004, Bert et al., 2005, Ha et al., 2007, Hsu et al., 2006, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989a, Tomasek and Tukac, 2007). The physicochemical properties of BFDV were first described by Ritchie et al. (1989b) and experimental infections were first carried out by Wylie and Pass (1987).

Other avian circoviruses or circovirus-like syndromes have since been identified and described in geese (*Anser anser*) (Todd et al., 2001c), pigeons and doves (Order *Columbiformes.*) (Coletti et al., 2000, Todd et al., 2008, Todd et al., 2001c), canaries (Phenix et al., 2001, Todd et al., 2001b), ducks
(Anas sp.) (Hattermann et al., 2003, Soike et al., 2004), common and spotless starlings (Sturnus vulgaris and Sturnus unicolor) (Johne et al., 2006a), Australian ravens (Corvus coronoides) (Stewart et al., 2006), Gouldian finches (Chloebia gouldiae) (Shivaprasad et al., 2004, Todd et al., 2007), black-backed, herring and black-headed gulls (Larus dominicanus, Larus argentatus and Larus ridibundus) (Smyth et al., 2006b, Todd et al., 2007, Twentyman et al., 1999) and mute swans (Cygnus olor) (Halami et al., 2008).

2.3.2 Virus characteristics

2.3.2.1 Physical
BFDV is a non-enveloped, icosahedral virus of approximately 17-20 nm diameter. Studies by various authors have revealed virions of between 12-15 nm (Ritchie et al., 1990) and 20-22 nm (Wylie and Pass, 1987) in diameter (Table 2.3). BFDV has been shown to haemagglutinate the erythrocytes of goffin cockatoos (Cacatua goffiní), guinea pigs (Ritchie et al., 1991b), galahs (Eolophus roseicapillus) (Raidal et al., 1993b), eastern long-billed corellas, sulphur-crested cockatoos, gang-gang cockatoos (Callocephalon fimbriatum), Major Mitchell’s cockatoos (Cacatua leadbeateri) (Soares et al., 1998), salmon-crested cockatoos (Cacatua moluccensis), umbrella cockatoos (Cacatua alba) and cockatiels (Sanada and Sanada, 2000), African grey parrots, brown-headed parrots (Poicephalus cryptoxanthus) (Kondiah et al., 2005) and some geese (Sexton et al., 1994). Interestingly, Soares et al. (1998) found that BFDV did not haemagglutinate erythrocytes from red-shouldered macaws (Ara nobilis), orange-winged Amazon parrots (Amazona amazonica), blue and gold macaws (Ara ararauna) and blue-fronted Amazon parrots (Amazona aestiva). These are all South American birds, which
suggests that there may be differences in the physical and pathogenic characteristics of different virus isolates or differences in host susceptibility between species.

Table 2.3: Electron micrographic studies of the virus by various authors

<table>
<thead>
<tr>
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<td>Ritchie et al. (1989)</td>
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<tr>
<td>Ritchie et al. (1990)</td>
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<td>Ritchie et al. (1991)</td>
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<td>Jacobson et al. (1986)</td>
<td>17-22</td>
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<tr>
<td>Pass and Perry (1985)</td>
<td>20</td>
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<tr>
<td>Latimer et al. (1991)</td>
<td>&gt;10</td>
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<tr>
<td>McOrist et al. (1984)</td>
<td>17-20</td>
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<tr>
<td>Kiatipattanasakul-banlunara et al. (2002)</td>
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</table>

2.3.2.2 Chemical
Purification of beak and feather disease virus by differential and isopycnic ultracentrifugation reveals a protein peak of between 1.35 g/mL (Raidal, 1994) and 1.378 g/mL (Ritchie et al., 1989b) in caesium chloride (CsCl) (Table 2.4).

Table 2.4: Beak and Feather Disease Virus buoyant density in caesium chloride (CsCl) as described by various authors

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<tr>
<td>Raidal 1994</td>
<td>1.35-1.37</td>
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2.3.2.3 Protein composition
Ritchie et al. (1989) reported that there are three major viral-associated proteins, of approximately 26.3 kDa, 23.7 kDa and 15.9 kDa molecular weight. The authors analysed purified viral preparations by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and found that the 26.3 kDa
and 23.7 kDa proteins were present in roughly equimolar amounts and comprised 88% of the total protein. In a subsequent publication, Ritchie et al. (1990) also identified two other proteins, one of 48 kDa and another of 58 kDa in size, which were present in small amounts.

2.3.2.4 Molecular
Initial studies using virus preparations from cockatoos characterised the viral genome as single stranded DNA, of approximately 1.7-2.0 kb in length (Ritchie et al., 1989b). Dideoxynucleotide sequencing of the DNA of 9 isolates of BFDV by Bassami et al. (2001) revealed that the genomes of these isolates ranged in size from 1993 nucleotides (BFDV-AUS and Galah-WA, GenBank Accession Numbers AF080560 and AF311298)) to 2018 nucleotides (BB-WA and LB-WA, GenBank Accession Numbers AF311295 and AF311296) (Table 2.5). Sequencing of the BFDV genome has revealed other putative ORFs which potentially code for proteins of 8.7 kDa or more (Bassami et al., 1998) (Tables 2.1, 2.5). Open reading frames 1 and 2 are similar to the replication-associated (rep) and capsid (cap) genes (respectively) of porcine circovirus (Todd et al., 1991). Open reading frames 3 to 7 did not share significant identity with any known genomic sequence in the GenBank sequence database. Other isolates examined in the same publication had between 4 and 7 ORFs coding for putative proteins greater than 8.7 kDa (GenBank).

Bassami et al. (1998) also identified a potential stem-loop structure with a nonanucleotide motif at the apex, thought to be the initiation site for RCR (Steinfeldt et al., 2001, Steinfeldt et al., 2006) and a twice-repeated octanucleotide motif, thought to be the binding site for Rep (Mankertz et al.,
2000, Steinfeldt et al., 2001) (Figure 2.4b). Studies by Heath et al. (2006) using different variants of baculovirus-expressed BFDV capsid proteins have identified an NLS within the first 41 amino acids of the N-terminal of the capsid protein.

BFDV has a high amount of genetic diversity. The nucleotide identity of full-length BFDV sequences has been reported to be between 84-97% (Bassami et al., 2001, Heath et al., 2004). The replication associated gene and protein are the most conserved, with nucleotide and amino acid identities of 86-99% and 87-98% (Bassami et al., 2001, Hess et al., 2004, Khalesi et al., 2005, Kondiah et al., 2006) respectively. The capsid-associated gene and protein are much more variable, with nucleotide and amino acid identities reported to be between 80-100% and 73-99% (Bassami et al., 2001, Raue et al., 2004) respectively.

Table 2.5: Genome size and predicted ORFs of nine isolates of Beak and Feather Disease Virus, adapted from Bassami et al. (1998)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Genome size</th>
<th>Total ORFs</th>
<th>ORFV1 (nt)</th>
<th>ORFC1 (nt)</th>
<th>ORFC2 (nt)</th>
<th>ORFV2 (nt)</th>
<th>ORFV3 (nt)</th>
<th>ORFC3 (nt)</th>
<th>ORFC4 (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFDV-AUS</td>
<td>1993</td>
<td>7</td>
<td>867</td>
<td>741</td>
<td>480</td>
<td>318</td>
<td>303</td>
<td>264</td>
<td>258</td>
</tr>
<tr>
<td>Galah-WA</td>
<td>1993</td>
<td>7</td>
<td>867</td>
<td>741</td>
<td>495</td>
<td>303</td>
<td>264</td>
<td>318</td>
<td></td>
</tr>
<tr>
<td>MMC-WA</td>
<td>1995</td>
<td>7</td>
<td>867</td>
<td>720</td>
<td>273</td>
<td>336</td>
<td>303</td>
<td>264</td>
<td>279</td>
</tr>
<tr>
<td>ELBC-SA</td>
<td>1992</td>
<td>5</td>
<td>867</td>
<td>729</td>
<td>495</td>
<td>303</td>
<td>264</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB-WA</td>
<td>2018</td>
<td>4</td>
<td>867</td>
<td>735</td>
<td>495</td>
<td>474</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB-WA</td>
<td>2018</td>
<td>4</td>
<td>867</td>
<td>735</td>
<td>456</td>
<td>474</td>
<td></td>
<td></td>
<td>297</td>
</tr>
<tr>
<td>LK-VIC</td>
<td>2007</td>
<td>5</td>
<td>870</td>
<td>741</td>
<td>498</td>
<td>474</td>
<td></td>
<td></td>
<td>282</td>
</tr>
<tr>
<td>SCC1-WA</td>
<td>1993</td>
<td>6</td>
<td>867</td>
<td>741</td>
<td>495</td>
<td>318</td>
<td>474</td>
<td></td>
<td>306</td>
</tr>
<tr>
<td>SCC-NT</td>
<td>1994</td>
<td>5</td>
<td>867</td>
<td>720</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>372</td>
</tr>
<tr>
<td>BFDV-USA</td>
<td>1993</td>
<td>4</td>
<td>897</td>
<td>732</td>
<td>471</td>
<td></td>
<td></td>
<td></td>
<td>474</td>
</tr>
</tbody>
</table>
2.3.3 Phylogenetics
Bassami et al. (2001) found that BFDV isolates grouped into genetic clusters and that the same isolates grouped together when different methods of analysis were used. Overall nucleotide identity of the isolates ranged from 84% to 97%, indicating some genetic variation. Ritchie et al. (2003) also found that isolates grouped into clusters and proposed cockatoo, budgerigar and loriikeet lineages of BFDV based on their sequence data. Genetic variation is also evident with restriction digestion. Albertyn et al. (2003) demonstrated different restriction enzyme digestion patterns between individual budgerigars (*Melopsittacus undulatus*) and between budgerigars and ring-neck parakeets (*Psittacula krameri*). Similarly, Kondiah et al. (2006) demonstrated 6 different restriction enzyme digestion patterns after *Hae III* digestion of 161 PCR-amplified *rep* fragments.
Analysis of a section of the capsid gene of 40 samples from a range of psittacine species by Raue et al. (2004) found that isolates did not group according to host species, but did find that isolates collected from an aviary with an outbreak of acute PBFD in lorikeets grouped together. Similarly, isolates from African grey parrots with leucopenia grouped separately to isolates from African greys with feather lesions. They suggested that the course of disease may be influenced by the characteristics of a particular BFDV strain and that strains may exist that preferentially infect a certain species. The difference in nonsynonymous polymorphisms between isolates may represent the accumulation of random mutations and account for these apparent differences (Nei, 2007). The existence of geographically distinct lineages has also been proposed (Heath et al., 2004, Kondiah et al., 2006); these authors also found that sequences organised into distinct groups according to species (cockatoo [Cacatua sp., Eolophus sp.], lorikeet [Trichoglossus sp.], budgerigar and lovebird [Agapornis sp.]). Given the high relative diversity of BFDV sequences and the large number of species within the Psittacidae, the existence of genotypes related to host species, pathogenicity or geographic location is to be expected. Given the many different strategies used to investigate the phylogenetics of the virus, amplification and analysis of whole sequences, cap or rep or fragments thereof, it is difficult to draw any consistent conclusions.

It is highly likely that all species of psittacine birds are susceptible to all strains of BFDV. However there have not been any studies thus far investigating whether this is the case or whether strains of BFDV cause appreciable
differences in the progression of disease. Of interest would be the
determination of whether a bird infected with virus isolated from an African
grey parrot, for example, was more likely to cause acute disease and
leucopenia or whether infection with virus isolated from lorikeets was more
likely to be associated with the development of protective antibody than
infection with a virus of cockatoo origin. It is also curious, given the wide
range of *Psittaciforme* species reported to be susceptible, that the cockatiel is
greatly underrepresented in both the scientific and lay literature. As yet, no
studies have investigated the possible mechanism behind this apparent
difference in susceptibility.

Most studies thus far have focused on *cap* as the main determinant of the
pathogenicity of the virus (Heath et al., 2004, Raue et al., 2004) but parts of
the sequence other than *cap* and *rep* contribute to pathogenicity. Work with
PCV2 showing that CpG motifs play a role in the modulation of IFN-α
expression (Hasslung, 2003) and as such CpG motifs may play a similar role
in modulating cytokines during the course of BFDV infection. Fenaux et al.
(2003) demonstrated that when the capsid-coding region of PCV2 was cloned
into the genomic backbone of PCV1, the resultant chimeric virus was less
pathogenic than wild-type PCV2. This is not to say that *cap* and the capsid
protein are not significant in the pathogenesis of the disease, simply that it is
not the sole determinant of pathogenesis.

A higher rate of genetic diversity has been found within *cap* (64.3% similarity
between isolates) compared to *rep* (93.2% similarity) (Heath et al., 2004) and
the bulk of this variation has been shown to occur within specific areas of the
capsid sequence. This may represent an attempt by the virus to evade the immune system, or may be the result of host-virus interactions specific to individual species. The only cross-reactivity study conducted thus far, by Khalesi et al., (2005) did not demonstrate the existence of serotypes using polyclonal antisera. However, some of the individual epitopes of the capsid protein which correlate to particular psittacine species may vary. Without a panel of monoclonal antibodies and virus from a range of species to conduct epitope mapping studies, though, this is impossible to establish.

In addition, the variation in genotypes and disease progression is not only dependant on the characteristics of the virus, but also on the immune response of the host, which will also vary between species. As evidence of this, recombination within BFDV genomes has been described (Heath et al., 2004, Hughes and Piontkivska, 2007) and Hughes and Pionkivska (2007) found that BFDV and other circoviruses were subject to past and ongoing purifying selection. The varied methods used to analyse sequences of BFDV isolates may severely compromise the value of the phylogenetic analyses, as analysis of genomic fragments ignores the effect of recombination (Posada and Crandall, 2001), so full-length gene and genome sequences should be used.

Various differences in antigenic composition, nucleotide sequences, replication kinetics and pathogenicity have been documented between PCV1 and 2 (Hughes and Piontkivska, 2007, Lefebvre et al., 2008, Mahe et al., 2000, Meehan et al., 1998) between strains of PCV2 (Lefebvre et al., 2008, Meerts et al., 2005a, Opriessnig et al., 2006d) and CAV (Lucio et al., 1990,
Nogueira et al., 2007) and TT viruses are divided into genogroups (Devalle and Niel, 2004, Niel et al., 2005, Peng et al., 2002). It is likely that such differences exist between BFDV isolates, but any proposed differences must be confirmed experimentally.

2.3.4 Pathophysiology

2.3.4.1 Clinical signs
There have been 2 distinct clinical syndromes described, an acute form which occurs in nestlings (Pass and Perry, 1985, Raidal and Cross, 1995) and African grey parrots (Doneley, 2003, Pass and Perry, 1985, Schoemaker et al., 2000) and a chronic form which occurs in many species of psittacine birds.

The acute form is characterised by the rapid development of hepatic necrosis, leucopenia and green diarrhoea after infection. The acute form of PBFD can be fatal, with clinical signs occurring in wild-caught nestling and fledgling cockatoos at a prevalence of up to 90% (Raidal and Cross, 1995). In contrast, African grey parrots often die within a week of the development of clinical signs (Schoemaker et al., 2000).

More commonly, PBFD develops as a chronic disease, characterised by progressive feather and beak dystrophy. Claw abnormalities occur occasionally, and generally develop well after feather and beak lesions (Perry, 1981) become apparent. Feather loss is roughly symmetrical, usually affecting the powder down feathers first (Pass and Perry, 1984), then flight and tail feathers before progressively involving the rest of the body until birds appear to be bald.
The pattern of feather loss is not consistent between birds, however, depending on the stage of moult that the bird is in when disease symptoms start. Normal feathers that fall out are replaced by dystrophic, abnormal feathers. The beak becomes progressively elongated, develops fracture lines and may eventually fracture or slough off. Chronically affected birds are predisposed to secondary infections (Pass and Perry, 1984, Ritchie et al., 1989a) and it is suspected that the virus is immunosuppressive. However, the precise mechanism of such immunosuppression is unknown.

2.3.4.2 Clinical pathology
The acute form of PBFD is associated with severe leucopenia in African grey parrots (Doneley, 2003, Schoemaker et al., 2000, Stanford, 2004). There has only been one report investigating the clinicopathologic changes in psittacines affected with the chronic form of PBFD (Jacobson et al., 1986). These authors did not find any consistent differences in haematologic or serum enzyme measurements between healthy and PBFD-affected cockatoos. They did, however, find that the birds with PBFD had consistently lower serum protein concentrations, characterised by low prealbumin and gamma globulin. Ritchie et al. (1989a) proposed that this may be an indication of the mechanism of immunosuppression caused by the virus.

2.3.4.3 Gross pathology
The most obvious external gross lesions are the loss of feathers and beak and claw abnormalities. The pattern of feather loss is variable, depending on the stage of moult when disease signs start. Dystrophic feathers may have
retained feather sheaths, blood within the feather shafts, curled and deformed feathers, stress lines evident in the vane, short clubbed feathers and feathers with a circumferential constriction. The beak may be overgrown, with transverse fracture lines and parts of the beak may fracture off or slough (Figure 2.5). Claw abnormalities are similar, but less consistent (Pass and Perry, 1985). Birds affected by the acute form of the disease will have feather lesions, but beak and claw abnormalities will typically not be evident, as they take longer to develop.

Gross internal findings may include bile staining within the proventriculus, ventriculus and intestine, the liver may appear swollen and mottled (Jergens et al., 1988) and there may be atrophy of the thymus and bursa of Fabricus (Pass and Perry, 1985, Raidal and Cross, 1995, Ritchie et al., 1989a).

### 2.3.4.4 Histopathology
Lesions within the skin and epidermis include multifocal epithelial cell necrosis, necrosis of distal pulp and haemorrhage into the distal shaft of feathers and epidermal hyperplasia and hyperkeratosis (McOrist et al., 1984, Pass and Perry, 1984, Wylie and Pass, 1987). There may also be infiltration of heterophils and lymphocytes into the pulp of some feathers. Basophilic intracytoplasmic inclusions can be found within macrophages in the feather pulp (Figure 2.5) and some epithelial keratinocytes may contain intracytoplasmic or intranuclear inclusions (Pass and Perry, 1984, Raidal and Cross, 1995). Within the beak, degeneration and necrosis of epithelial cells occurs in the basal and intermediate cell layers. Chronic beak lesions are also
associated with inflammation as a result of the presence of bacteria within exudate and the keratinised layers of epithelium (Pass and Perry, 1984).

The liver may be congested, with multifocal areas of necrosis of varying severity (Raidal and Cross, 1995). Characteristic basophilic inclusions may be present in Kupffer cells within the liver and intranuclear inclusions may be present within epithelial cells of collecting ducts and tubules. The thymus and bursa may show varying degrees of atrophy and necrosis. Focal aggregates of necrotic lymphocytes often contain macrophages with typical inclusions and necrotic lymphocytes with intranuclear inclusions may also be visible (Pass and Perry, 1984, Raidal and Cross, 1995). Intracytoplasmic inclusion bodies within macrophages are variable in size and shape and electron microscopic examination shows that they are composed of particles 17-20 nm in diameter arranged in a paracrystalline array (McOrist et al., 1984, Pass and Perry, 1984, Wylie and Pass, 1987).
2.3.4.5 Pathophysiology

**Transmission** – The virus is excreted via the feathers and the gastrointestinal tract. Consequently, high concentrations of virus can be detected in liver tissue, bile, crop secretions, faeces and feather dander (Raidal and Cross, 1994b, Ritchie et al., 1991a, Ritchie et al., 1991b). Infection is most likely by oral and/or intracloacal ingestion of virus, as demonstrated by experimental infection studies (Wylie and Pass, 1987). Experimental infection can also be accomplished by intramuscular administration of the virus (Raidal and Cross, 1995, Raidal et al., 1993a, Wylie and Pass, 1987).
BFDV is strongly suspected to be transmitted vertically (Rahaus et al., 2008, Ritchie et al., 1994). Both CAV and PCV2 may be transmitted vertically, via semen (Hoop, 1992, Hoop, 1993, McIntosh et al., 2006) and in utero (Cardona et al., 2000) and BFDV DNA has been amplified from embryonated eggs (Rahaus et al., 2008). However, vertical transmission of BFDV has been extremely difficult to prove, due to the difficulty in finding BFDV-free aviaries and the difficulty involved in ruling out horizontal transmission to the embryo via cloacal secretions and nesting material.

**Progression of infection** – The virus is epitheliotrophic (Latimer et al., 1991, McOrist et al., 1984, Pass and Perry, 1984, Ramis et al., 1994, Ritchie et al., 1989b) and primary sites of replication include the skin (including the epithelium of the beak and claws), liver, gastrointestinal tract and bursa of Fabricus (Raidal and Cross, 1994b, Wylie and Pass, 1987). The virus replicates to high titres in these tissues and also spreads to the spleen, thyroid, parathyroid and bone marrow (Latimer et al., 1990). The incubation period of the virus is unknown, as the appearance of clinical signs depends on the stage of moult of the bird. Experimentally infected nestlings show signs of acute PBFD from 21-28 days post-infection (Raidal and Cross, 1995, Raidal et al., 1993a). However, as the disease progresses, feather and beak dystrophy occur as described above and chronically affected birds may become immunosuppressed. Immunosuppression as a result of BFDV infection is highly likely, as lymphoid atrophy and necrosis has been demonstrated histologically (Pass and Perry, 1984, Raidal and Cross, 1995) and chronically affected birds commonly excrete large amounts of virus as
measured by HA with no detectable antibodies measurable by HI (Khalesi et al., 2005).

Interferon-α-modulatory CpG sequences have been described in PCV2 (Hasslung et al., 2003) and likely also exist within BFDV. These oligodeoxyribonucleotides (ODNs) have been shown to have both inhibitory and stimulatory effects on the induction of IFN-α and an inhibitory effect on the production of TNF-α in NIPCs (Hasslung et al., 2003, Stevenson et al., 2006, Wikstrom et al., 2007), independent of viral replication or the presence of Cap. Other research into PCV2, profiling serum cytokine expression, cytokine mRNA levels in lymphoid tissues and PBMCs in cell culture, suggests a T-cell mediated immunosuppression under the influence of IL-10 (Darwich et al., 2003a, Darwich et al., 2003b, Darwich et al., 2008, Kekarainen et al., 2008, Stevenson et al., 2006) and the viral genome and ORFC2 protein may be involved in this (Hasslung et al., 2003, Liu et al., 2006b, Liu et al., 2007b, Wikstrom et al., 2007).

Individuals within some species of birds may recover from clinical PBFD. For example, lorikeets (Trichoglossus sp.), Eclectus parrots (Eclectus sp.) and king parrots (Alisterus scapulatus) often develop protective HI titres and excretion of virus ceases. In contrast, other species appear highly susceptible; cockatoos (Cacatua sp.) typically develop the chronic form of the disease, with high HA titres detectable in feathers and faeces and no detectable HI titre. African grey parrots are known to be highly susceptible to the virus, typically developing the acute form of the disease. In contrast, there are no published reports of PBFD in cockatiels. There is only one published report of
BFDV in a cockatiel, diagnosed by PCR (Khalesi et al., 2005). However, this diagnosis was made from a feather submitted to the laboratory by a third party and the identity of the bird was not confirmed. Variations in nucleotide sequences, antigenic composition, replication kinetics and pathogenicity probably exist between BFDV isolates, as is the case with PCV1 and strains of PCV2 (Hughes and Piontkivska, 2007, Lefebvre et al., 2008, Mahe et al., 2000, Meehan et al., 1998, Meerts et al., 2005a, Opriessnig et al., 2006d), but this is yet to be investigated.

2.3.4.6 Epidemiology
Horizontal transmission via faeces, feather dander and crop secretions is likely the major means by which the virus is spread (Hess et al., 2004, Raidal and Cross, 1994b, Ritchie et al., 1991a). Vertical transmission is suspected but not confirmed, as has been discussed previously (Section 2.3.4.5). All species of psittacine birds are considered susceptible and individual serotypes were not found in the one cross-reactivity study (Khalesi et al., 2005) undertaken.

There are reports of BFDV infection worldwide in both wild (BusinessWorld, 1996, Ha et al., 2007, Kock et al., 1993, McOrist et al., 1984, Raidal et al., 1993b) and captive (Albertyn et al., 2004, Bert et al., 2005, Ha et al., 2007, Hsu et al., 2006, Kiatipattanasakul-Banlunara et al., 2002, Perry, 1981, Rahaus and Wolff, 2003) psittacines. McOrist et al. (1984) reported the presence of clinical signs of PBFD in 10-20% of sulphur-crested cockatoos in 3 wild flocks in Australia. The reported prevalence rates of viral DNA vary between 14.8-28.0% in wild flocks (Ha et al., 2007) and between 8.05-83.90%
in captive flocks (Bert et al., 2005, Khalesi et al., 2005, Rahaus and Wolff, 2003). Seroprevalences of between 16% and 62% (Khalesi et al., 2005, Raidal and Cross, 1994a) among captive flocks and seroprevalence of between 41% and 94% has been reported in wild flocks (Raidal et al., 1993b).

2.3.5 Diagnosis, treatment and prevention

2.3.5.1 Diagnosis
 Diagnosis of PBFD can be made on clinical signs alone, but signs can be confused with other diseases of the skin and feathers (see below). Different methods that have been used for the detection of BFDV include histology, electron microscopy (EM), haemagglutination (HA), immunohistochemistry (IHC), DNA in-situ hybridisation (ISH), polymerase chain reaction (PCR) and real-time PCR. The serological detection of anti-BFDV antibodies has been conducted by haemagglutination-inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) assays.

**Histology** – Histology is used to detect viral inclusion bodies in tissue samples. Typical histological lesions include multifocal epithelial cell necrosis, necrosis of distal pulp and haemorrhage into the distal shaft of feathers and epidermal hyperplasia and hyperkeratosis. There may also be scattered infiltrates of inflammatory cells within feather follicles. Characteristic basophilic botryoid intracytoplasmic inclusions can often be seen within macrophages and the nuclei of necrotic epithelial cells may be swollen with basophilic inclusions (Jacobson et al., 1986, McOrist et al., 1984, Pass and Perry, 1984, Raidal and Cross, 1995, Wylie and Pass, 1987). The principal advantage of histology is that identification of the characteristic inclusion bodies is
diagnostic for BFDV infection. Disadvantages include the relative lack of sensitivity, as inclusion bodies can be difficult to detect and the need for a surgical procedure to collect tissue samples.

**Electron microscopy** – EM has been used by many authors for the detection of viral particles, using preparations from faeces, feathers, skin, GIT and liver. Electron microscopic examination of these preparations demonstrates virus-like particles 17-20 nm in diameter. Intracytoplasmic inclusion bodies within macrophages are variable in size and shape and electron microscopic examination shows these virus-like particles arranged in a paracrystalline array (McOrist et al., 1984, Pass and Perry, 1984, Wylie and Pass, 1987). The advantages of EM are that it allows more detailed analysis of viral inclusions and measurement of viral particles. Disadvantages include the need to identify viral inclusions by histology first and the extra cost and complexity involved in sample preparation and analysis.

**DNA in-situ hybridisation** – Latimer et al. (1994) used a 30-base oligonucleotide to detect viral DNA in the skin, feathers, liver, spleen, gastrointestinal tract, trachea and lungs, kidney and myocardium. The advantages of DNA in-situ hybridisation are its high sensitivity and specificity and the ability to identify the subcellular location of viral particles within specific tissues. Disadvantages include the need for specialised reagents and the extra cost and complexity involved in sample preparation and analysis.
**Haemagglutination** – HA is used to detect viral antigen. BFDV was shown by Ritchie et al. (1991b) to be a haemagglutinating virus, and a standardised HA assay was developed by Raidal et al. (1993c). HA activity can be detected in a variety of tissues such as feathers, faeces and liver but feathers are the most commonly used tissue. The advantages of HA include its use as a non-invasive ante mortem diagnostic test and the ability to detect viral antigen in a wide range of tissues. The main disadvantages include the need for erythrocytes from suitable birds, virus purified from the feathers of infected birds and polyclonal antibody preparations. Other disadvantages include the variation inherent in these reagents and the need for specialised training to perform and interpret the assay.

**Haemagglutination inhibition** – HI is used for the detection of antibodies to BFDV in serum and plasma samples. Polyclonal anti-BFDV serum inhibits viral haemagglutination (Raidal et al., 1993c, Ritchie et al., 1991b). Raidal and Cross (1993c) developed an HI assay for routine diagnostic use and this assay has been implemented in various serological studies (Khalesi et al., 2005, Raidal and Cross, 1994a, Raidal and Cross, 1995, Raidal et al., 1993a, Raidal et al., 1993b). Advantages and disadvantages are similar to the HA assay.

**Enzyme-linked immunosorbent assay** – Only one indirect ELISA for the detection of antibodies to BFDV in psittacine sera has been developed (Johne et al., 2004). However, this assay has not yet been validated with a large number of samples and uses a secondary antibody directed against IgY from an African grey parrot. Since the cross-reactivity between the IgY of different
psittacine birds is not known, it is impossible to guarantee the validity of the assay when used with sera from other species. Another indirect ELISA has been described (Ritchie et al., 1992), however it was only used to screen mouse sera and hybridoma supernatants during the development of anti-BFDV monoclonal antibodies. Advantages of ELISA include its use as a non-invasive ante mortem diagnostic test and the ability to screen multiple samples simultaneously. The main disadvantage is the need for specialised reagents and equipment to perform the assay.

**Immunohistochemistry** – Various authors have described immunohistochemical techniques for the detection of BFDV antigens in histological sections (Latimer et al., 1990, Latimer et al., 1991, Raidal and Cross, 1995, Ritchie et al., 1992). Most of these use polyclonal antibodies raised in rabbits or chickens; Ritchie et al. (1992) used monoclonal antibodies raised against virus purified from the skin and feathers of diseased birds. The advantages of immunohistochemistry are its high sensitivity and specificity and the ability to identify the subcellular location of viral particles within specific tissues. Disadvantages include the need for specialised reagents and the extra cost and complexity involved in sample preparation and analysis.

**Polymerase chain reaction (PCR) and real-time PCR** – A variety of PCR techniques have been described for the detection of BFDV DNA, from standard polymerase chain reaction (Albertyn et al., 2004, Ritchie et al., 2003, Ypelaar et al., 1999) to nested PCR (Kiatipattanasakul-Banlunara et al., 2002) and real-time PCR (Raue et al., 2004). A duplex shuttle PCR assay has also been developed to differentiate between BFDV and avian polyomavirus (APV)
Of these, only the method developed by Ypelaar et al. (1999) has been validated with a sufficiently large number of samples. PCR using degenerate primers has been used to identify novel circoviruses of starlings (Johne et al., 2006a), ducks (Hattermann et al., 2003), geese (Todd et al., 2001c), canaries (Phenix et al., 2001, Todd et al., 2001b) and ravens (Khalesi et al., 2005). Eisenberg et al. (2003) also used a standard PCR assay to detect circovirus DNA in the tissues of ostrich (*Struthio camelus*) chicks. The advantages of PCR assays are their high sensitivity and specificity and the ability to test multiple samples at once. Disadvantages include the need for specialised reagents and training to perform the assays and the potential for sample contamination due to the high sensitivity of the assays.

**Multiply-primed rolling circle amplification (RCA)** – RCA is a technique which uses random hexamer primers to amplify circular DNA templates (Rector et al., 2004). It can be used to amplify DNA from plasmids, bacteriophages and viruses with circular DNA genomes such as papillomaviruses, polyomaviruses and circoviruses. It has been used to identify novel papillomaviruses in manatees (Rector et al., 2004), polyomaviruses in a Eurasian bullfinch (*Pyrrhula pyrrhula griseiventris*) and a Eurasian jackdaw (*Corvus monedula*) (Johne et al., 2006b), a novel circovirus in starlings (Johne et al., 2006a), human TTV and both genogroups of Sd-TTV (Niel et al., 2005). The advantages of RCA include its high sensitivity and the ability to detect circoviral DNA from a range of avian species. The main disadvantage is the need for specialised reagents and training to perform the assays.
2.3.5.2 Treatment
Treatment is mainly supportive, as no reliable specific treatment is currently available. Birds affected with chronic BFDV can live for many years, even after the development of feather and beak lesions but often succumb to chronic infections. Cytokines show promise for the treatment of many viral diseases, Stanford (2004) used chicken IFN-γ successfully to treat seven of ten African grey parrots affected with acute BFDV and IFN-α has been used to treat patients co-infected with hepatitis C and TTV (Akahane et al., 1999, Chayama et al., 1999, Hagiwara et al., 1999). Interferon of avian origin is not yet commercially available. Consequently, the efficacy of interferon in the treatment of chronic cases is yet to be investigated, especially in light of findings that IFN-γ enhances PCV2 replication in cell culture (Meerts et al., 2005a) and may have a deleterious effect on the development of protective immunity (Blanchard et al., 2003).

Tomasek and Tukac (2007) claimed successful treatment of BFDV-infected birds with β-(1,3/1,6)-D-Glucan, but their experimental design was poor – an insufficient number of birds was treated, no control group was included and no evidence was provided that absence of BFDV DNA was not simply due to the development of an appropriate antibody response. Even though the compound has been shown to enhance immune responses in chickens (Cheng et al., 2004, Guo et al., 2003) and may actually be useful in the treatment of BFDV, no reliable conclusions can be drawn from this study. RNA interference (RNAi) has been used successfully to inhibit PCV2 viral DNA replication and protein synthesis in vitro (Sun et al., 2007) and in
experimentally infected mice (Liu et al., 2006a). RNA interference may therefore be useful for treatment of BFDV infection and further testing is warranted.

2.3.5.3 Prevention and control
Prevention of BFDV in an avicultural setting is difficult and frustrating. The virus is highly infectious and resistant to the extremes of temperature and various chemicals (Raidal and Cross, 1994b). Strict quarantine and screening of new additions to the flock, using a combination of PCR, HA and HI tests to detect infected birds, is recommended. The source of birds is also important. Birds at pet shops or areas where many birds are mixed are more likely to be infected and infection may be difficult to detect, depending on which tests are selected. Khalesi et al. (2005) showed that in a flock of 56 peach-faced lovebirds (*Agapornis roseicollis*), of the 47 birds that were PCR-positive on blood samples, only 10 were also positive on feather samples.

Prevention of access to the flock by wild birds is important, as is prevention of contamination of the flock with faeces from wild birds. Stringent hygiene protocols should be in place, including regular cleaning with an appropriate disinfectant and control of movement between quarantined birds and the flock. Since BFDV cannot be propagated *in vitro*, the relative effects of various disinfectants cannot be evaluated. However, disinfection using peroxide compounds (Virkon S) has been recommended by the Department of Environment and Heritage, Australia, for use in captive breeding programs of endangered psittacine species (Cross, 2006). Virkon S at a 1% solution has
been shown to inactivate non-enveloped viruses and bacterial spores, so in theory it is the only suitable disinfectant.

Currently there is no commercially available vaccine for BFDV. An experimental inactivated vaccine has been shown to be effective (Raidal and Cross, 1994a, Raidal et al., 1993a), but since virus purified from infected birds is required, the vaccine is time consuming, expensive and ethically questionable to produce. As no cell culture system has been developed to grow the virus successfully in vitro, recombinant techniques show the most promise for the development of effective vaccines that may be produced on a large scale. Recombinant capsid proteins, expressed in mammalian, bacterial and insect-cell based systems have been proposed for use in diagnostic tests and vaccines for BFDV (Heath et al., 2006, Johne et al., 2004, Khalesi et al., 2005, Stewart et al., 2007), PCV (Liu et al., 2004, Nawagitgul et al., 2002, Zhou et al., 2005) and GoCV (Scott et al., 2006).

DNA vaccines and recombinant virus vectors also have the potential to be effective vaccines. DNA vaccines have been developed against many aetiological agents including viruses (Cheung et al., 2004, Li et al., 2004, Wang et al., 2006, Yang et al., 2004), bacteria (Ko et al., 2005) and parasites (Lillehoj et al., 2005, Nagata et al., 1999, Wu et al., 2004) and virus-vectored vaccines providing protection against animal diseases are already commercially available (Minke et al., 2007, Pardoa et al., 2007, Poulet et al., 2003). However, since the vector used in these vaccines is a canarypox virus, an alternate viral vector would need to be developed for use in avian patients. In theory, apart from the great difficulty and expense of harvesting native
BFDV for vaccine production, recombinant vaccines are theoretically better than vaccines made from whole inactivated virus. This is because the modulation of immune memory is independent of viral infectivity (Kekarainen et al., 2008, Vincent et al., 2007). Thus, the presence of the viral genome and key proteins in preparations of inactivated virus may encourage viral persistence after natural infection post-vaccination and may reduce the immune response to other pathogens.
Chapter 3. A Quantitative, Real-Time Polymerase Chain Assay for Beak and Feather Disease Virus (BFDV)

3.1 Introduction and Aims

3.1.1 Introduction
PCR-based assays for the detection of BFDV DNA are in widespread use throughout the world. Quantitative real-time PCR assays generally have a low limit of detection and have the advantages over standard PCR assays in that they do not require post-reaction processing to visualise PCR products and they can quantify the amount of DNA present in a sample. BFDV agglutinates the erythrocytes of many birds (Kondiah et al., 2005, Raidal and Cross, 1994b, Ritchie et al., 1991b, Sanada and Sanada, 2000, Sexton et al., 1994, Soares et al., 1998), which has allowed the development of HA and HI assays for the detection of virus and anti-BFDV antibodies respectively (Raidal et al., 1993c).

The ambisense viral genome contains two major coding regions. ORFV1 encodes the replication-associated protein and ORFC1 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 (Raufe 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 novel qPCR assay for the detection and characterisation of BFDV infection.

3.1.2 Aims
The aims of this experiment were to develop and validate a quantitative, real-time PCR assay in order to investigate the kinetics of the acute phase of BFDV infection in both vaccinated and non-vaccinated psittacine birds.

3.2 Materials and Methods
3.2.1 Experimental design
The independent and dependent variables were time and viral load, measured in both blood and feather extracts, respectively. When sample types were compared to determine their suitability for use in a diagnostic assay, blood viral load was the independent variable and feather viral load was the dependent variable. For the calculation of diagnostic sensitivity and specificity, the results from the qPCR assay were compared to the results of the HA and standard PCR assays, both of which were conducted by Mr Nicolai Bonne. Insofar as was practical, the qPCR was developed and validated in accordance with guidelines established by the Office International des Epizooites (O.I.E.) for the development of PCR methods for the diagnosis of infectious diseases (Belak and Thoren, 2004, Jacobson, 2004). Assay
validation did not include the assessment of reproducibility between laboratories or the continued testing of new samples over time, as these were beyond the scope of this experiment. The vaccination trial comprised samples from 18 eastern long-billed corellas, 13 vaccinated and 5 non-vaccinated, plus samples from 2 galahs. Therefore, at each sample collection there were 15 replicates from vaccinated birds and 5 replicates from non-vaccinated birds and the experiment had an unbalanced design. Samples were collected at 5 time points.

3.2.2 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, galahs, rainbow lorikeets (*Trichoglossus haematodus*), purple crowned lorikeets (*Glossopsitta porphyrocephala*), eclectus parrots (*Eclectus roratus*), budgerigar, Indian ringneck (*Psittacula eupatria eupatria*), Jardine’s parrot (*Poicephalus gulielmi*), green-cheeked conure (*Pyrrhura molinae*), sun conure (*Aratinga solstitialis*), yellow-tailed black cockatoo (*Calyptorhynchus funereus*) and cockatiel. After DNA was extracted as described below in Section 3.2.4, samples were stored at -20°C for up to 5 years.

To validate the qPCR, the DNA extracts of 108 blood samples from eastern long-billed corellas vaccinated with a recombinant BFDV capsid protein (Bonne et al., 2008b, Stewart et al., 2007) 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. The vaccination trial was conducted in collaboration with Mr Nicolai Bonne. Mr Bonne also performed the HA and standard PCR assays, the results of which were used in the validation of the 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.

3.2.3 Birds and vaccination
Nestling galahs and eastern long-billed corellas were provided by the Western Australian Department of Environment and Conservation (DEC). Nestlings were removed from the nesting sites of Carnaby’s white-tailed black cockatoos (*Calyptorhynchus latirostris*) as part of an existing conservation program. Nestlings were kept in sibling groups, housed in temperature-controlled incubators (Brinsea) and fed a commercial hand-rearing formula (Roudybush). Blood samples were collected as described above in Section 3.2.2 on presentation and at weekly intervals thereafter until the vaccine trial started. Mr Nicolai Bonne performed PCR and HI assays on the initial samples and PCR assays on the subsequent samples. Birds were considered BFDV-free if they tested negative on presentation and at three consecutive time points thereafter. When birds were being weaned they were transferred to individual wire cages in a separate room, then transferred to a free-flight
area within that room once weaned. After weaning, the birds were maintained on a diet of commercial parrot pellets (Passwell, Wombaroo) and fresh fruit and vegetables. Strict quarantine was maintained during hand rearing, weaning and vaccination.

The recombinant protein was expressed and purified as described by Stewart et al. (2007) and below in Section 5.2.2. Vaccination and challenge were conducted as described by Bonne et al. (2008b). Briefly, the vaccine was prepared by emulsifying equal volumes of the recombinant protein solution with Freund's incomplete adjuvant (Sigma Aldrich) prior to administration. Vaccinated birds (n = 15) received 10 µg of the recombinant protein by intramuscular (IM) injection on day 0 of the trial, followed by 66.8 µg of the protein on day 11. Non-vaccinated birds (n=5) received no inoculum. Mr Nicolai Bonne administered a challenge dose of 0.5 mL of live BFDV to all birds on day 27. Birds received 0.4 mL of virus suspension IM and 0.1 mL per os (PO). The virus suspension used for challenge was originally prepared by Associate Professor Shane Raidal (Raidal et al., 1993a) and had been stored at -20°C for 13 years. The HA titre of the suspension was determined by Mr Nicolai Bonne to be log212 HAU/50 µL. Feathers and blood were collected on days 0, 11, 27, 40, 47, 53, 68 and 124 as described above in Section 3.2.2 and processed as described below in Sections 3.2.4 and 3.2.5, respectively.

3.2.4 Extraction of DNA from feathers
Viral DNA was extracted from feathers using the methods described by Ypelaar et al. (1999). Five millimetres of feather calamus were aseptically cut
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 added and the tube again vortexed. 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 were also made using feather eluates prepared for HA testing. Feathers were incubated with 100 µL phosphate buffered saline (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 and then boiled at 100ºC for 10 minutes.

3.2.5 Extraction of DNA from dried blood spots:
Viral DNA was extracted from dried blood spots with the Qiagen blood mini kit (Qiagen), using a modified dried blood spot protocol. Three spots of blood, each approximately 5mm in diameter, that had been applied to and dried on filter paper, were cut out using scissors or a hole punch and collected in a microcentrifuge tube (Eppendorf). Scissors and hole punches were disinfected between samples using the methods described by Bonne et al.
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 85°C for 10 minutes, then centrifuged. Twenty microlitres of proteinase K (Qiagen) were then added to the tube, the tube vortexed and then incubated at 56°C for 1 hour. After incubation, the solution was centrifuged and DNA extracted from the supernatant according to the manufacturer’s protocol.

3.2.6 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 and P6 (Table 3.1) (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 (Eppendorf). The optimised PCR reactions consisted of 2 mM MgCl₂, 5 µL of 5x polymerisation buffer containing mixed deoxynucleotide triphosphates (dNTPs), 3.34 µM SYTO9 fluorescent dye (Invitrogen), 12.8 pmol of each primer and 0.1 U 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 sequential dilutions of a synthetic oligonucleotide, BFDV qST (Table 3.1) (Geneworks). The oligonucleotide was diluted to an initial concentration of 1x10¹² copies/µL.
and then serially diluted 1:100 to give standards of $1 \times 10^{10}$, $1 \times 10^8$ and $1 \times 10^6$.

The $1 \times 10^6$ standard was serially diluted 1:10 to give standards of $1 \times 10^5$, $1 \times 10^4$, $1 \times 10^3$, $1 \times 10^2$ and the $1 \times 10^2$ standard diluted 1:2 to give a standard of 50 copies/µL. Two reactions using ultrapure water instead of DNA were used as negative controls in each run.

Table 3.1: Oligonucleotides used for the quantitative, real-time PCR.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real P5</td>
<td>5' GGA CGC AAA ATG AAG GAA G 3'</td>
<td>491-509</td>
</tr>
<tr>
<td>Real P6</td>
<td>5' TAG CGA GAG GTT ATG CAA GC 3'</td>
<td>552-571</td>
</tr>
<tr>
<td>BFDV qST</td>
<td>5' ATG TCC GGA CGC AAA ATG AAG</td>
<td>485-577</td>
</tr>
<tr>
<td></td>
<td>GAA GTC GCA GCT GAA TTC CGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAT TCC TAC GTC AGG CAT GGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGT GGC TTG CAT AAC CTC TCG CTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTG GTT 3'</td>
<td></td>
</tr>
</tbody>
</table>

Reactions were carried out in a Corbett Rotor-gene 3000 (Corbett Research) real-time thermocycler. Cycling conditions are outlined in Table 3.2.

Table 3.2: Cycling conditions used for the quantitative, real-time PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.7 Melt curve analysis
A melt curve analysis was performed after each run, with the mixture being cooled to 60°C for 45 seconds then heated in one-degree increments to 95°C.

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 80°C and 85°C; the melting temperature of the synthetic oligonucleotide was 82.3°C.

3.2.8 Quantitation of viral load
The threshold cycle (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.

3.2.9 Statistical analysis
Calculation of r-squared values of standard curves was performed using the software supplied with the Rotor-Gene. Calculation of the coefficient of variation of the standards, the standard error of groups of replicates and the sensitivity, specificity and positive and negative predictive values of the assay (Jacobson, 2004) was performed using Microsoft Excel. Calculation of the lines of best fit and the correlation between blood and feather viral loads was performed using CoPlot (CoHort Software, 2005).

3.3 Results
3.3.1 A 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. Negative control samples were consistently negative.
The assay successfully detected BFDV DNA in the blood of all control (non-vaccinated) corellas (Figure 3.1a). 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 1,177 ± 723 copies/µL (range 209 to 4,057 copies/µL) by 6 weeks post challenge (Figure 3.1a). Specific peaks were present in the melt curves of BFDV-positive samples between 82 – 84.5°C.

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 1,000 copies/µL, as the coefficient of variation of the $10^3$ and $10^2$ standards was close to 100% 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 (Figure 3.1b). 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 detected in the feather extracts of 3 vaccinated birds at 2 weeks post-challenge, 6 vaccinated birds at 4 weeks post-challenge and 10 vaccinated birds at 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 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) (Figure 3.2).
Figure 3.2: 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.

Two sets of comparisons were made for the calculation of sensitivity and specificity. Firstly, similar tests were compared. For blood samples, the standard PCR assay was considered the gold standard assay and was used for comparison with the results of the qPCR assay using blood samples. For feather eluates, the HA assay was considered the gold standard assay and was used for comparison with the results of the qPCR assay 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.

3.4 Discussion
Given the high prevalence of BFDV infection worldwide, sensitive and specific detection methods which use standard methods and consistent reagents are both highly desirable and necessary. The qPCR assay described here has a very low limit of detection and can be used to quantify the amount of virus present in the blood and in feather preparations. As such, it should prove 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.
Although it is well accepted that qPCR assays have a much greater analytical sensitivity than standard PCR (Mackay, 2004, Wong and Medrano, 2005), currently the standard PCR assay is the gold standard for the detection of BFDV nucleic acid and hence the comparative diagnostic sensitivity and specificity were determined. 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 negative by standard PCR assay. However, because of this fact the analytical sensitivity could not be determined precisely. Importantly, in this study it was impossible to differentiate between true positive results below the limit of detection of the HA and standard PCR assays and false positive results. In this study, the effect is likely to be minimal since there was accurate information on the infection status of the birds. Ongoing comparisons between the qPCR assay and existing assays using birds of known infection status will still be necessary to elucidate the true analytical sensitivity and specificity.

The comparative diagnostic sensitivity and specificity was good, however because of the greater analytical sensitivity of the qPCR, 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 within values accepted by the OIE (Jacobson, 1998); reproducibility could not be assessed since testing in other laboratories was impractical. Negative predictive values were uniformly high 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. The effect of false positive results cannot be ignored, however, but remains to be elucidated. 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. Not only did the qPCR assay detect viral DNA in birds that tested negative by the standard PCR (Bonne et al., 2008b), 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 et al., 2008b, Shearer et al., 2009) indicates that vaccination does not prevent viral replication. This is a common scenario with other vaccines
(Opriessnig et al., 2006c) 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 vertically infected despite the presence of antibodies in the parents, as occurs with PCV2 and CAV (Brentano et al., 2005, Larochelle et al., 2000). 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. Prevention of vertical infection would be an important measure in reducing the incidence of PBFD and consequent secondary infections over a bird’s lifespan. This information would also be most valuable for the management of conservation programs for endangered psittacine birds and non-endangered captive flocks alike. Of course, maternal antibody levels will wane soon after hatching and so horizontal infection is still probably the primary route of infection.

The main advantages that this qPCR assay has over existing PCR assays are 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 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 1,000 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 were 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 is also more important when testing for cell-associated virus and this study was concerned with the detection of
circulating and excreted virus. 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. Appropriate control genes for qPCR assays used to study BFDV will require studies to account for disease states, the tissue(s) sampled and the species of host psittacine (Li et al., 2005, Maccoux et al., 2007, Radonić et al., 2005, Robinson et al., 2007, Spinsanti et al., 2006, Sturzenbaum and Kille, 2001, Tang et al., 2007, Tricarico et al., 2002).

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 may be advantageous, as feathers used for BFDV PCR assays typically vary in size and the quality of pulp material between growing and mature feathers varies dramatically. In practice, however, as long as sample volumes are similar between assays the extra cost and complexity of data normalisation would probably outweigh the benefits.

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. The dye SYTO9 was chosen for use in this assay in preference to SYBR green, as the melting curves are highly reproducible over a greater range than with 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 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 3.1b) is similar to those of viral load in blood samples of vaccinated and non-vaccinated birds (Figure 3.1a), 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. Although these results were accurate in that they detected the presence of the virus, they did not accurately represent the infection or health status of the birds and this is an important fact to remember when making a diagnosis of infection or disease using only the results of PCR-based assays.
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 pathogen(s) 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 ID$_{50}$ 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., 2008a) 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 of HA and HI assays (Johne et al., 2004). However, in regards to PCR assays, 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 also introduce unacceptable levels of variation and 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, present as either neutralised viral particles or free genomic DNA, may be present in blood for up to 3 months (Lazizi and Pillot, 1993) and whole 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.
Chapter 4. Beak and Feather Disease Virus (BFDV) Infection in Cockatiels (*Nymphicus hollandicus*)

4.1 Introduction and Aims

4.1.1 Introduction

Beak and feather disease virus (BFDV) is the most common viral infection of psittacine birds and the chronic debilitating feather disease that it causes has been confirmed in more than 60 psittacine species. Thus all psittacine birds are highly likely to be susceptible (Albertyn et al., 2004, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989b, Tomasek and Tukac, 2007). Surveys have been carried out in both wild and captive psittacine populations and reported virus prevalence rates vary between 10-94%, depending on the method of detection (Ha et al., 2007, Khalesi et al., 2005, McOrist et al., 1984, Rahaus and Wolff, 2003, Raidal et al., 1993b). Given the wide range of *Psittaciforme* species reported to be susceptible, it is curious that the cockatiel is greatly underrepresented in both the scientific and lay literature. Indeed we know of no published reports of PBFD in cockatiels even though the species is one of the most commonly kept companion bird species worldwide. It seems the only evidence of BFDV infection occurring in the cockatiel was a diagnosis made by polymerase chain reaction (PCR) in our own laboratory (Khalesi et al., 2005).

In order to investigate the apparently low rate of BFDV infection in cockatiels a survey of cockatiels at 3 commercial aviaries in Perth, Western Australia was conducted using PCR, as well as HA and HI assays. In addition, BFDV
DNA samples from the feathers of 2 cockatiels submitted to us for BFDV diagnostic testing were amplified by PCR, sequenced and analysed.

4.1.2 Aims
This experiment aimed to determine whether cockatiels are susceptible to BFDV infection. If cockatiels were found to be susceptible to infection by BFDV, the additional aims of this experiment were to determine the prevalence of infected birds and to assess the differences between BFDV isolates from cockatiels and other psittacine species.

4.2 Materials and Methods
4.2.1 Experimental design
When investigating the existence and prevalence of BFDV infection in cockatiels, the identity of individual birds was the independent variable and the assay (HI, HA or PCR) result was the dependent variable. When conducting cross-reactivity assays, the source of the antibodies and the antigen were the independent variables and the HI titre was the dependent variable. In the analysis of the nucleotide sequences of the PCR-positive cockatiels, the identity of the individual bird was the independent variable and the sequence length and characteristics were the dependent variables. For the comparison of the BFDV sequences from the cockatiels with the BFDV sequences from other psittacine birds, the bird species was the independent variable and the CpG density, pairwise distances (nucleotide and amino acid) and phylogenetic grouping, bootstrap support and posterior probability were the dependent variables.
4.2.1 Samples
A survey of cockatiels in commercial aviaries was also conducted. Blood and feathers from 88 cockatiels at three commercial aviaries that had a laboratory confirmed history of housing PBFD-affected psittacine birds were taken for testing. Samples were collected as described in Section 3.2.2.

Feathers from two additional cockatiels (isolates 05-106 and 05-726) with characteristic skin lesions consistent with PBFD and that had tested BFDV-positive by PCR and HA were used for subsequent PCR analysis and DNA sequencing as described below. A formalin-fixed feather follicle skin biopsy, submitted along with the feather of 05-106, was also processed by routine histology methods, stained with haematoxylin and eosin and examined by light microscopy for the presence of characteristic inclusion bodies (Fig 5.1a) and BFDV infection was confirmed by IHC (Fig 5.1b) as described below.

4.2.2 Immunohistochemical (IHC) staining
Immunohistochemistry was performed on tissue sections from case 05-106 as described in Section 5.2.5.4, except that antigen retrieval was not carried out and the monoclonal antibody was used at the optimal 1:500 dilution in TBS.

4.2.3 Extraction of DNA from feathers
Viral DNA was extracted from feathers of the two cockatiels 05-106 and 05-726 using the methods described by Ypelaar et al. (1999). Five millimetres of feather calamus were aseptically cut and placed into a microcentrifuge tube
(Eppendorf). To this, 200 µL of 70% (v/v) ethanol were added and the tube briefly vortexed. The ethanol was then removed, 200 µL of sterile distilled water 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 MgCl$_2$, 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 37°C for one to two hours, before being heated to 95°C 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.

4.2.4 Polymerase chain reaction
Polymerase chain reaction was carried out using methods similar to those described by Ypelaar et al. (1999). Primers P2 and P4 (Table 4.1) amplify a 717 bp fragment of ORF V1 of BFDV DNA and were used to test the surveyed cockatiels. Cockatiels 05-106 and 05-726 had previously tested positive for BFDV DNA using these two primers. Primers SeqP5 and SeqP10 (Table 4.1) were designed to amplify a 1479 bp fragment, the ends of which overlapped with the fragment generated by primers P2 and P4. Cockatiels 05-106 and 05-726 were tested using both primer sets; the two overlapping amplicons included the entire BFDV genome and were used in subsequent sequencing reactions. The PCR reaction consisted of 2 mM MgCl$_2$, 5 µL of 5x polymerisation buffer containing dNTPs, 12.8 pmol of each primer, 0.1 U of Tth-Plus DNA polymerase and made to a total volume of 25 µL using ultrapure water (all reagents Fischer Biotec). Reactions were carried out in an
Eppendorf Mastercycler Gradient thermocycler (Eppendorf). The PCR reaction using primers SeqP5 and SeqP10 was identical, except that 3 mM MgCl$_2$ was used and the annealing temperature was lowered to 48°C. Cycling conditions are outlined in Table 4.2. PCR products were visualised on a 1% agarose gel with the addition of 0.001% ethidium bromide, run at 90 V for 30 minutes.

Table 4.1: Primers used for the amplification and sequencing of Beak and Feather Disease Virus DNA isolated from cockatiels.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2 (forward)</td>
<td>5’ AAC CCT ACA GAC GGC GAG 3’</td>
<td>182-199</td>
</tr>
<tr>
<td>P4 (reverse)</td>
<td>5’ GTC ACA GTC CTC CTT GTA CC 3’</td>
<td>879-898</td>
</tr>
<tr>
<td>Seq P5 (forward)</td>
<td>5’ CTG CGA CCG TTA CCC ACA TA 3’</td>
<td>781-800</td>
</tr>
<tr>
<td>Seq P10 (reverse)</td>
<td>5’ TCG CCC TTT TCC CGT CCA AC 3’</td>
<td>248-267</td>
</tr>
</tbody>
</table>

Table 4.2: Cycling conditions used for the amplification and sequencing of Beak and Feather Disease Virus DNA isolated from cockatiels.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>60 (P2P4)/48 (SeqP5SeqP10)</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.2.5 Haemagglutination (HA) assay
Haemagglutination assays were carried out as described by Raidal et al. (1993c). Feathers were incubated with 100 µL PBS at 60°C for 1 hour in a microcentrifuge tube (Eppendorf). The suspension was centrifuged briefly after incubation and 50 µL of the supernatant were added to 50 µL PBS in a microtitre plate (Eppendorf). Serial dilutions of the solution were made by removing 50 µL of the solution from the first well, mixing with 50 µL PBS in the
next well and repeating the process across the row of the microtitre plate. Negative and positive control lanes were included for each batch of samples tested. The negative control consisted of PBS only and the positive control was a 50 µL suspension of virus purified from feathers and organs of a BFDV-infected sulphur-crested cockatoo, diluted to give a HA titre of 4-8 HAU/50 µL. To each well 50 µL of a 0.75-0.85% (v/v) solution of type A galah erythrocytes were then added and the microtitre plate incubated for 1 hour at 37ºC. A positive HA titre was indicated by suspension of the erythrocytes in the well of the microtitre plate, whereas a negative HA result was indicated by the sedimentation of erythrocytes within the well.

4.2.6 Haemagglutination-inhibition (HI) assays
Haemagglutination-inhibition assays were carried out as described by Raidal et al. (1993c). The antigen preparation consisted of virus purified from the feathers of a sulphur-crested cockatoo with PBFD, diluted to give a HA titre of 4-8 HAU/50 µL. Plasma, serum or dried blood spots on filter paper were used for testing. Plasma or serum was first heat-inactivated at 57ºC for 30 minutes, then 100 µL serum were added to 1 mL of 5% (w/v) acid-washed kaolin and the mixture incubated for 1 hour at room temperature, or overnight at 4ºC. Equal volumes of the supernatant were then haemadsorbed against a 10% (v/v) suspension of normal (non-haemagglutinating) galah erythrocytes for 18 hours at 4ºC.

For blood collected onto filter paper one 0.5 cm diameter spot of blood on filter paper was cut out using scissors or a hole punch and collected into a
microcentrifuge tube (Eppendorf). One hundred microlitres of 5% (w/v) acid-washed kaolin were added and serum eluted from the paper by incubating the mixture for 1 hour at room temperature, or overnight at 4°C. Then 50 µL of the supernatant were haemadsorbed against 50 µL of a 10% (v/v) suspension of normal (non-haemagglutinating) galah erythrocytes for 18 hours at 4°C.

For testing, 50 µL of haemadsorbed serum were added to 50 µL PBS in a microtitre plate well (Eppendorf). Serial dilutions of the solution were made by removing 50 µL of the solution from the first well, mixing with 50 µL PBS in the next well and repeating the process across the row of the microtitre plate. Negative and positive control lanes were included for each batch of samples tested. The negative control was a 50 µL suspension of purified virus, diluted to give a HA titre of 4-8 HAU/50 µL. The positive control was a serial dilution of 50 µL of chicken anti-BFDV polyclonal sera, processed as above. A positive HI titre was indicated by the sedimentation of the erythrocytes within the well of the microtitre plate, whereas a negative HI result was indicated by the suspension of the erythrocytes in the well.

4.2.7 Cross-reactivity assays
An additional set of HI assays was performed to investigate the possible existence of a cockatiel-specific BFDV serotype. HI assays were performed by Mrs Margaret Sharp as described above, except that the antigen and negative control was virus eluted from the feather of a cockatiel that had tested positive for BFDV by PCR and HA. Sera from seven different psittacine species including two short-billed corellas, a sulphur-crested cockatoo, two
rainbow lorikeets, one long-billed corella, one red lory and one galah–corella hybrid with known HI titres were reacted against virus eluted from cockatiels 05-106 and 05-726. Sera from all cockatiels sampled at the commercial aviaries were tested against BFDV eluted from the feather of cockatiel 05-106 and the polyclonal anti-BFDV used as a positive control was also tested against a more dilute (2 HAU/50 µL) sample of the same virus.

4.2.8 Nucleotide sequence determination and analysis
PCR products were purified using an Axyprep PCR cleanup kit (Axygen), according to the manufacturer’s instructions. Dideoxynucleotide sequencing was carried out using an ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions, except that the reaction volume was reduced to 10 µL and the annealing temperature used when sequencing the 1478 bp products was reduced to 50ºC. Sequencing reactions consisted of 2 µL of reaction buffer (containing Tris-HCl, MgCl₂, fluorescently-labelled dNTPs and AmpliTaq DNA polymerase, concentrations not supplied; Perkin Elmer), 1 µL of 5x sequencing buffer (composition not supplied, Perkin Elmer), 10-20 ng of 717 bp PCR product or 20-40 ng of the 1479 bp PCR product, then made up to a total of 10 µL with ultrapure water (Fischer Biotec).

DNA sequences were determined using an Applied Biosystems 3730 DNA Analyser and edited using Sequence Scanner v1.0 (Applied Biosystems) and GeneTool Lite (BTI Software). Edited sequences were analysed using MEGA 3.1 (Kumar et al., 2004) and MrBayes (Ronquist and Huelsenbeck, 2003).
Neighbour-joining (NJ), maximum parsimony (MP) and Bayesian trees were constructed with 1,000 bootstrap cycles for NJ and MP trees. Sequences used for construction and analysis of phylogenetic trees are listed in Appendix 1.

4.2.9 Statistical analysis
Pairwise distances between the sequences derived from cockatiels and sequences derived from other psittacine birds and bootstrap support for NJ and MP trees were calculated using Mega 3.1 (Kumar et al., 2004). For Bayesian analysis of phylogeny, posterior probabilities were calculated using MrBayes (Ronquist and Huelsenbeck, 2003).

4.3 Results
4.3.1 Survey of cockatiels for evidence of BFDV and cross-reactivity assays
Of the 88 cockatiels tested using primers P2 and P4, none were positive for BFDV by PCR or HA and none had detectable antibodies to BFDV. None of the cockatiels surveyed had detectable HI activity against BFDV eluted from a cockatiel feather. Six of the 8 known anti-BFDV HI positive sera tested inhibited HA eluted from the feather of cockatiel 05-106 and similarly 5 of the 8 sera inhibited HA eluted from isolate 05-726. The polyclonal chicken anti-BFDV antibody used in the cross-reactivity assays did not inhibit agglutination by either cockatiel virus isolate (Table 4.3). When the polyclonal chicken anti-BFDV antibody was tested against a more dilute sample of the same virus, however, some HI activity was observed (Figure 4.1).
Table 4.3: Haemagglutination inhibition cross-reactivity of known positive anti-Beak and Feather Disease Virus (cockatoo) psittacine sera against virus eluted from the feathers of two cockatiels with Psittacine Beak and Feather Disease.

<table>
<thead>
<tr>
<th>Antibody Source</th>
<th>Cockatoo BFDV</th>
<th>Cockatiel 05-106</th>
<th>Cockatiel 05-726</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken anti-BFDV</td>
<td>+</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Red Lory</td>
<td>+</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Long-billed Corella</td>
<td>+</td>
<td>+</td>
<td>neg</td>
</tr>
<tr>
<td>Galah/Corella hybrid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rainbow lorikeet</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rainbow lorikeet</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Short bille corella</td>
<td>+</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>Short bille corella</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulphur crested cockatoo</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 4.1: Haemagglutination-inhibition assay using a dilute (2 HAU/50 µL) sample of Beak and Feather Disease Virus eluted from the feather of cockatiel 05-106 as the antigen. Row 1: A back-titration of the virus; row 2: polyclonal chicken anti-BFDV antibody, raised against virus purified from feathers and organs of a BFDV-infected sulphur-crested cockatoo (*Cacatua galerita*).

4.3.2 Analysis and sequencing of PCR-positive samples from cockatiels

Characteristic basophilic intracytoplasmic inclusions were visible on haematoxylin and eosin-stained skin sections from cockatiel 05-106 (Figure 4.2a). Immunohistochemical staining revealed large amounts of BFDV capsid antigen in the nucleus and cytoplasm of epidermal cells (Figure 4.2b).

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

Figure 4.2: A. Histological section of feather follicle from cockatiel 05-106, showing characteristic basophilic inclusions within macrophages in the feather pulp (arrow heads). Bar=0.5µm B. Immunohistochemical stain of the same section showing strong positive (brown) reaction to Beak and Feather Disease Virus antigen throughout the epidermis. Bar = 50 µm.
4.3.3 Comparison of nucleotide sequences of BFDV isolated from cockatiels with other known BFDV sequences

4.3.3.1 Full-length sequences
Pairwise distances between cockatiel sequences and 26 other BFDV sequences varied between 3.2-15.5% (05-106) and 3.8-14.5% (05-726) at the nucleotide level and between 3.8-19% (05-106) and 5.2-19.2% (05-726) at the amino acid level. Neighbour joining phylogenetic analysis showed that both cockatiel sequences (isolate 05-106 and 05-726 GenBank Accession Nos. EF457974 & EF457975, respectively) clustered within cockatoo and galah isolates, however maximum parsimony (not shown) and Bayesian analysis grouped the cockatiel isolates distinctly separately, with a high posterior probability (Figure 4.3). Bayesian analysis also identified 3 distinct clades within the sequences (Figure 4.3): the cockatiels comprised clade number 1; the cockatoos and galahs plus 3 African grey parrots (AY521236, AY450443 and AY450435), a white-bellied caique (AY450434) and a Cape parrot (AY450439) made up clade 2 and the remaining clade was made up of all Agapornis sp. isolates, the remaining African grey parrot and Poicephalus sp. plus a single rainbow lorikey and a single Indian ringneck isolate.
Figure 4.3: Bayesian phylogram constructed using full-length Beak and Feather Disease Virus nucleotide sequences demonstrating the distance between cockatiel (*Nymphicus hollandicus*) BFDV sequences (EF457974 & EF457975) in relationship to published BFDV sequences isolated from other *Psittacidae* and *Cacatuidae*. Numbers at the nodes indicate posterior probability.
A plot of CpG islands within the cockatiel sequences showed high proportions of CpG motifs throughout most of the sequence. Gardiner-Garden and Frommer (1987), defined a candidate CpG island as having a Y-value of >0.6 on the CpG plot and a GC content of >50%. As such, the first 200 bases of the sequence, then bases 240-650, 680-1160, 1210-1380 and 1760-1993 of the viral strand contain candidate CpG islands. The complementary strand had candidate CpG islands within the first 30 nt and between nt 420-600, 640-1120, 1150-1570 and 1575-1993 (Figure 4.4).

Particular points of interest are spikes in the CpG plot at nt 280-400, 672, 1167 and 1660-1880 of the viral strand and nt 30-360, 630, 1120 and 1330-1540 of the complementary strand (Figure 4.4a). The spikes at nt 672, 1167 and 1660-1760 of the viral strand and nt 630, 1130 and the first spike between nt 30-360 of the complementary strand are less likely to be significant, though, as the GC content in these regions is less than 50%. The whole sequence was GC-rich, as the GC content was only less than 50% between bases 200-240, 650-680, 1160-1210 and 1380-1760 of the viral strand and nt 60-400, 580-640, 1120-1145 and 1550-1600 of the complementary strand (Figure 4.4b). Comparison of CpG plots derived from the cockatiel isolates demonstrated subtle differences compared to plots derived from published BFDV sequences from a sulphur-crested cockatoo (AF080560), rainbow lorikeet (AF311299) and peach-faced lovebird (AF311296). GC density plots for these isolates demonstrated minimal variation between the 4 isolates.
Figure 4.4: A (i and ii): CpG plots of the complementary and viral strand, respectively, of the cockatiel (*Nymphicus hollandicus*) 05-106 isolate, overlain on respective CpG plots of Beak and Feather Disease Virus (BFDV) isolates from a sulphur-crested cockatoo (*Cacatua galerita*), rainbow lorikeet (*Trichoglossus haematodus*) and peach-faced lovebird (*Agapornis roseicollis*). B (iii and iv): GC plots of the complementary and viral strand, respectively, of the same isolates. The CpG plots demonstrate variability at nt 1700 in the viral strand and at nt 250 in the complementary strand. GC density in these regions is > 0.5.
4.3.3.2 ORFV1
Sequences of both cockatiel isolates had a start codon of ATG located at position 131. The stop codon for both isolates was TGA, located at nt 997. The predicted size of ORFV1 was 867 nt. Distances between cockatiel sequences and other BFDV sequences varied between 2.5-11.9% (05-106) and 2.4-11.1% (05-726) at the nucleotide level and between 2.2-11% (05-106) and 1.1-10.4% (05-726) at the amino acid level. Neighbour-joining and maximum parsimony phylogenetic analysis showed that both cockatiel sequences clustered closest to, but separate from, cockatoo and galah isolates (Figure 4.5).

4.3.3.3 ORFC1
Sequences of both cockatiel isolates had a putative start codon at nt 16 (CTG) of the complementary strand (or nt 1978 of the viral strand), as per Bassami et al. (2001). The stop codon for both isolates was a TAA at nt 757 of the complementary strand (nt 1235 of the viral strand). Distances between cockatiel sequences and other BFDV sequences varied between 2.0-18.9% (05-106) and 5.9-19.1% (05-726) at the nucleotide level and between 6.0-28.5% (05-106) and 6.8-27.4% (05-726) at the amino acid level. Neighbour-joining and maximum parsimony phylogenetic analysis showed that both cockatiel sequences clustered within a clade of cockatoo and galah isolates (Figure 4.6). Alignment of translated amino acid sequences showed that 121 of 260 amino acids were conserved across all the isolates examined and 133 of 260 were variable. The areas between aa 68-83, 94-97 and 228-241 were especially variable but the significance of this is unknown.
Figure 4.5: Phylograms constructed using the nucleotide sequence encoding the replicase-associated protein of various Beak and Feather Disease Virus (BFDV) isolates. A: Neighbour-joining phylogram, B: Maximum parsimony phylogram. The distance between cockatiel (*Nymphicus hollandicus*) BFDV sequences (EF457974 & EF457975) in relationship to published BFDV sequences isolated from other *Psittacidae* and *Cacatuidae* is demonstrated. Note that the cockatiel BFDV sequences cluster separately to other BFDV isolates.
Figure 4.6: Phylograms constructed using the nucleotide sequence encoding the capsid protein of various Beak and Feather Disease Virus (BFDV) isolates. A: Neighbour-joining phylogram, B: Maximum parsimony phylogram. The distance between cockatiel (Nymphicus hollandicus) BFDV sequences (EF457974 & EF457975) in relationship to published BFDV sequences isolated from other Psittacidae and Cacatuidae is demonstrated. Note that the cockatiel BFDV sequences cluster within a group of BFDV isolates from members of the Cacatuidae.
4.4 Discussion
The data presented in this chapter provides histological, DNA sequence and serotyping evidence of BFDV infection in cockatiels, thus confirming that the species is susceptible to BFDV infection. Immunohistochemical staining and DNA sequence data and CpG analysis demonstrated antigenic and genetic relationship with BFDV isolates obtained from other Psittaciformes. However, maximum parsimony and Bayesian analysis of the cockatiel isolates placed them into a clade genetically distinct from other BFDV sequences and HI cross-reactivity analysis also demonstrated evidence of antigenic variation in one of the cockatiel BFDV isolates when it was used as the antigen against known positive BFDV antisera.

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

The low reported incidence of BFDV infection in cockatiels and our negative serological survey results are somewhat puzzling for such a supposedly common and infectious virus to which all Psittaciformes are presumed to be susceptible. It is highly unlikely that the negative PCR results of all birds surveyed represent a failure of the test. Khalesi et al. (2005) reported only 1 false negative PCR result from a total of 623 samples tested. The authors also showed that the PCR test detected the cockatiel BFDV isolate and this was confirmed in this study. It could be that all of the cockatiels we surveyed in this present paper were naïve to infection, and truly antibody negative. However, they were from commercial aviaries that had a high turn-over of a wide variety of psittacine bird species many of which we knew from clinical observations were expressing clinical signs of PBFD. Typically the birds in such establishments have a high incidence of BFDV infection, PBFD and a high HI antibody prevalence with budgerigars and lovebirds (Agapornis spp.) having the highest rates of infection (Khalesi et al., 2005).
According to published epidemiologic data an expected seroprevalence of 30-40% would be a conservative estimate (Raidal et al., 1993a, Raidal & Cross 1994b; Khalesi et al., 2005) of the expected seroprevalence within the population of cockatiels that we sampled and a sample size of 88 should have provided a 95% level of confidence of the estimate of the prevalence of infection (Thrushfield 1986). Failure to detect evidence of antibody in such a sample size provides strong evidence (95% confidence level) that the seroprevalence in the population of cockatiels sampled was less than 5% (Thrushfield 1986). This is a very low figure in comparison with other Psittaciforme species.

The lack of documented cases of cockatiels with PBFD in the literature along with the negative PCR and serological results obtained in our survey could be interpreted as evidence that cockatiels are somewhat innately resistant to BFDV infection. However, the PCR results in individual cockatiels reported here and by Khalesi et al. (2005) along with the histological evidence of BFDV infection (Figure 4.2) indicates that the species is susceptible to BFDV. Furthermore, the PCR results, DNA sequence analysis and HI cross-reactivity data provide evidence of a cockatiel-adapted BFDV which may be sufficiently different, genetically and antigenically, to most other BFDV isolates to be considered a separate strain. This is not surprising given evidence that avian circoviruses have coevolved with their host species (Ritchie et al., 2003; Stewart et al., 2006). Assessment of the phylogeny of Psittaciforme birds using mitochondrial DNA has placed the monotypic Nymphicus more closely
related to the black cockatoos (*Calyptorynchus & Callocephalon*) and not the
more distantly related white cockatoos (*Cacatua & Eolophus*) as was once
thought (Brown & Toft, 1999) and the results presented in this present paper
provide further support to this hypothesis. It is assumed that all species of
psittacine birds are susceptible to all isolates of BFDV, however to date there
have been limited studies investigating the infectivity of BFDV isolates (Raidal
et al., 1993a, Wylie and Pass, 1987) and none comparing the relative
infectivity and pathogenicity of different isolates. Experimental confirmation of
the infectivity of the cockatiel BFDV and comparison of the pathogenicity of
this isolate with other BFDV isolates would be desirable but may be hard to
accomplish given the difficulty in finding BFDV-free birds.

Given the data presented in this present study, there are some aspects of
BFDV evolution and epidemiology that are difficult to explain. Based on the
phylogenetic analysis (Figure 4.3), the cockatiel BFDV sequences are
genetically distinct from those obtained from other psittacine species.
However, the same method of analysis and other similar studies have also
found similar distinct genetic differences between BFDV isolates from
lovebirds, lorikeets and cockatoos. In addition, cross-reactivity work using
BFDV eluted from the feathers of two rainbow lorikeets, a musk lorikeet
(*Glossopsitta concinna*), a red lory, two swift parrots, a sulphur-crested
cockatoo and a scarlet chested parrot and anti-BFDV antibodies from the
same panel of birds as in this study has shown these not to be serologically
distinct (Khalesi et al., 2005). Therefore, specific mutation or genetic variation
in the cockatiel sequences cannot be clearly associated with any biological or antigenic characteristics.

Phylogenetic analysis of full length sequences and rep sequences grouped the cockatiels separately to other isolates, but analysis of cap sequences alone grouped the cockatiel sequences appropriately within a cockatoo clade (data not shown). Most studies thus far have focused on cap as the main determinant of the pathogenicity of the virus (Heath et al., 2004, Raue et al., 2004). However, the fact that cap grouped within a known clade while rep and full length sequences grouped separately suggests three likely possibilities. Firstly, that rep may have other functions than first thought. Secondly, parts of the sequence other than cap and rep play a part in pathogenesis. Thirdly, host factors (such as the presence or absence of cell surface receptors for virus attachment or major histocompatibility complex (MHC) presentation) play a significant role.

The second of these possibilities is supported by work with PCV2 showing that CpG motifs play a role in the modulation of IFN-α expression (Hasslung, 2003) and as such CpG motifs may play a similar role in modulating cytokines during the course of BFDV infection. Fenaux et al. (2003) demonstrated that when the capsid-coding region of PCV2 was cloned into the genomic backbone of PCV1, the resultant chimeric virus was less pathogenic than wild-type PCV2. Furthermore, Hughes and Piontkivska (2007) found a greater proportion of nonsynonymous polymorphisms within PCV2 rep, which are likely to be associated with random mutations that have affected the
pathogenicity of the virus. Recombination also plays a role in the genetic reassortment of the Circoviridae (Biagini et al., 2001, He et al., 2007, Heath et al., 2004, Ma et al., 2007, Olvera et al., 2007, Worobey, 2000). The effect of these random mutations, recombination and ongoing purifying selection probably do account for the apparent antigenic and pathogenic differences between BFDV isolates (Nei, 2007). This may even lead to the eventual existence of distinct strains, pathotypes or even serotypes, as has been suggested for PCV2 and CAV (Lucio et al., 1990, Meerts et al., 2005a, Nogueira et al., 2007, Opriessnig et al., 2006d) and as such it is unlikely that the full spectrum of genetic, antigenic and pathogenic differences will ever be mapped.

This is not to say that cap and the capsid protein are not significant in the pathogenesis of the disease. Mahe et al. (2000) identified capsid epitopes unique to PCV1 and PCV2 and the presence of unique epitopes may occur with BFDV as well and may explain the variable cross-reactivity of the cockatiel isolates. Considering that there are currently no cell-culture techniques or any in vitro methods to propagate BFDV, the identification of these unique epitopes and other motifs suspected to play a role in pathogenesis will need to be carried out using such techniques as epitope mapping, infectivity studies using infectious clones and analysis of nucleotide sequence polymorphisms. Likewise, infectivity studies with various BFDV isolates or mutant BFDV infectious clones and a wide range of psittacine species may be the only way to investigate the extent to which the genotype of an isolate affects its infectivity and pathogenicity. Such studies have been
applied to the porcine circoviruses (Hughes and Piontkivska, 2007, Lefebvre et al., 2008, Mahe et al., 2000, Meehan et al., 1998, Meerts et al., 2005a, Opriessnig et al., 2006d) and the VP2 protein of CAV (Wang et al., 2007a) and will no doubt be applicable to BFDV.
Chapter 5. Development of a Monoclonal Antibody to a Recombinant Beak and Feather Disease Virus (BFDV) Capsid Protein; its Applications in Western Blotting, Immunohistochemistry, Haemagglutination-Inhibition and the Development of a Novel Blocking Enzyme-Linked Immunosorbent Assay (bELISA)

5.1 Introduction and Aims

5.1.1 Introduction
The development of diagnostic assays for the detection of BFDV antigens and anti-BFDV antibodies has traditionally been hampered by the difficulty associated with producing suitable reagents, namely purified virus and polyclonal antibodies. At present, HA and HI assays (Raidal et al., 1993c, Ritchie et al., 1991b) are commonly used for the detection of virus excreted from feathers or faeces and anti-BFDV antibodies in psittacine sera, respectively. They require erythrocytes from suitable birds and virus purified from the feathers of infected birds. Polyclonal antibody preparations and variations in these reagents make consistency between tests difficult to achieve.

Similarly, methods for the detection of the virus in formalin-fixed, paraffin-embedded tissue, or in western- or dot-blots have relied on polyclonal antibodies (Johne et al., 2004, Latimer et al., 1990, Latimer et al., 1991, Raidal and Cross, 1995, Ritchie et al., 1990), making consistency between techniques difficult to achieve. Given that no cell culture system has been found to grow the virus successfully in vitro, recombinant proteins and monoclonal antibodies are the most attractive options for the development of standardised diagnostic assays. To this end, a recombinant BFDV capsid protein (Stewart et al., 2007) was used as the antigen source for the
development of a monoclonal antibody (MAb) specific to the capsid protein of BFDV and its application in western blotting, immunohistochemistry, ELISA and HI investigated.

Surveys using PCR-based assay methods have found virus prevalence rates of between 8% (Bert et al., 2005) and 39% (Rahaus and Wolff, 2003). The reported seroprevalence varies between 16% and 62% (Khalesi et al., 2005, Raidal and Cross, 1994a) among captive flocks and between 41% and 94% in wild flocks (Raidal et al., 1993b). Currently, an HI assay (Raidal et al., 1993c, Ritchie et al., 1991b) is the only method available for the detection of antibodies against BFDV in psittacine sera. This assay is useful since it detects both IgM and IgG antibodies from a wide range of species of psittacine birds. However it may suffer from an appreciable amount of inter-test variation due to variability in quality and quantity between virus preparations and the sensitivity to the virus of the erythrocytes used in the test. To overcome these limitations, the baculovirus-expressed recombinant BFDV capsid protein (Stewart et al., 2007) and monoclonal antibody raised against this protein (Shearer et al., 2008b) were used to develop a novel blocking (or competitive) ELISA (bELISA) for the detection of anti-BFDV antibodies. Blocking ELISAs have also been developed to detect aetiological agents (Hochel et al., 2004, Rauer and Conrad, 2001, Shurley et al., 2005) and the suitability of the bELISA for the detection of BFDV antigen was also investigated.
5.1.2 Aims
This principal aim of this experiment was to investigate the immune response to experimental BFDV infection in vaccinated and non-vaccinated psittacine birds using an assay which had a lower limit of detection and was more consistent than the existing HI assay. This necessitated production of a suitable antigen and antibody for use in the assay. Thus, the secondary aims of this experiment were to produce a recombinant BFDV capsid protein and monoclonal antibody against this protein and to describe their respective characteristics and applications.

5.2 Materials and Methods

5.2.1 Experimental design
During protein production, quantitation and characterisation, protein amount was the independent variable and pixel density and HA titre were the dependent variables. During monoclonal antibody production, quantitation and characterisation, mouse ID and MAb dilution factor were the independent variables and antibody responses (HI titre, ELISA titre and intensity of western blot and IHC intensity) were the dependent variables. When testing the birds using the bELISA, time was the independent variable and HI titre was the dependent variable. When determining the variability of the bELISA and comparing the bELISA and HI assays, replicate number, dilution factor and HI titre were the independent variables and deviation from mean PI and bELISA PI were the dependent variables. The experimental design of the vaccination trial is described in Section 3.2.1.
5.2.2 Production and purification of recombinant BFDV capsid protein

5.2.2.1 Protein production
A full-length, baculovirus-expressed BFDV capsid protein was expressed and purified as described by Stewart et al. (2007). Stored P5 (passage 5) virus stocks of known infectivity (>10^7 pfu/mL) (Stewart et al., 2007) were used to infect Sf9 insect cells at an MOI (multiplicity of infection) of 0.2 in a 100 mL volume of insect cell culture at a density of 2x10^6 cells/mL. Infected cells were then grown for 5 days at 28°C to express the recombinant capsid protein. After the growth period, the cell cultures were centrifuged at 1,700 rpm for 10 minutes at 4°C to pellet the cells. Cell pellets were stored at -80°C if the protein was not immediately purified.

5.2.2.2 Protein purification using Ni-NTA resin
Pellets of insect cells, produced as described above were lysed by adding lysis buffer (25 mM Tris-HCl, pH 8.5, 2.5 mM β-mercaptoethanol, 12.5 mM KCl, 0.5 mM PMSF, 0.5 U/mL DnaseI) and the cell pellet and lysis buffer solution mixed for 30 minutes at 4°C. The mixture was then sonicated on ice for 1 minute, 3 times in total, with a 1 minute interval between each sonication. Efficient lysis was checked using a haemocytometer, then cell debris was removed from the lysed cell mixture by ultracentrifugation at 10,000 g for 10 minutes at 4°C.

The supernatant, containing soluble protein, was then added to 1 mL of Ni-NTA resin (Qiagen) which had been pre-equilibrated with lysis buffer. The proteins were allowed to bind to the resin overnight at 4°C, whilst being mixed constantly. After binding, the liquid was allowed to flow through the column and was collected. The resin was then washed three times by inverting end-over-end for 20 minutes at 4°C with wash buffer (25 mM NaH$_2$PO$_4$, 250 mM NaCl, 5 mM Imidazole) and the liquid
collected after each wash. After washing, the protein was eluted by passing four 500 µL aliquots of elution buffer (25 mM NaH$_2$PO$_4$, 250 mM NaCl, 300 mM Imidazole) over the resin and collecting the liquid. The 2 mL of elution buffer was then re-added to the resin and the mixture allowed to stand at 4ºC overnight. The liquid was then collected and a further 2 elutions were made. The collected fractions were stored at 4ºC for short-term use or -20ºC/-80ºC for long-term storage, before being analysed as described below. All fractions were then analysed by SDS-PAGE electrophoresis and concentrations determined by densitometer analysis, in comparison with bovine serum albumin (BSA) and lysozyme standards.

5.2.2.3 Protein purification using ultracentrifugation
Insect cells were produced and lysed as described above. Cell pellets were resuspended in 4 mL PBS, then added to 750 µL of a 40% (w/v in PBS) sucrose cushion and centrifuged in an ultracentrifuge (Beckman Coulter) in an SW55Ti rotor at 28,000 rpm for 2 hours at 4ºC. Cell lysates, in 4 mL aliquots, were similarly treated, then the supernatants discarded and the cell pellets resuspended in 2 mL PBS and sonicated on ice for 10 seconds. Three millilitres of CsCl solution (3.8 M in distilled H$_2$O) was then added to the resuspended cell pellet and the solution centrifuged in an ultracentrifuge (Beckman Coulter) in an SW55Ti rotor at 29,000 rpm for 2 hours at 4ºC. Fractions were collected in 500 µL aliquots and then dialysed overnight in PBS at 4ºC. The collected fractions were then stored at 4ºC for short-term use or -20ºC/-80ºC for long-term storage, before being analysed as described below. Dialysed fractions were then analysed and quantified as described above.
5.2.2.4 Concentration of protein stocks
After dialysis, fractions containing the purified recombinant protein were dialysed in a 5-20% (w/v) solution of polyethylene glycol, MW 20,000 kDa (PEG 20K). Once the solution had decreased to the desired volume, the solution was removed, the dialysis tubing rinsed thoroughly in deionised water and the solution was collected and stored at 4ºC for short-term use or -20ºC/-80ºC for long-term storage.

5.2.3 Detection and analysis of baculovirus-expressed recombinant BFDV capsid protein
Purified recombinant proteins were analysed by SDS-PAGE, western blotting, haemagglutination assay, haemagglutination inhibition assay, transmission electron microscopy and protein sequencing.

5.2.3.1 SDS-PAGE
Protein samples were loaded onto a 12.5% (w/v) resolving gel containing 0.1% (w/v) SDS and subjected to electrophoresis at 200 V for 45-60 minutes. Gels were then stained with a solution of 45% (v/v) methanol, 5% (v/v) glacial acetic acid and 0.1% (w/v) Coomassie Brilliant blue (Bio-Rad) for at least 4 hours at room temperature with gentle rocking. Gels were then de-stained with a solution of 40% (v/v) methanol and 5% (v/v) glacial acetic acid for at least 4 hours at room temperature with gentle rocking, then dried and stored. Coomassie staining was not carried out on gels used for western blotting.
5.2.3.2 Western blotting
An aliquot of the recombinant protein was run on a 12.5% SDS-PAGE gel, then transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad). The membrane was then blocked with a solution of tris-buffered saline with 0.05% (v/v) Tween 20 (TBST) plus 5% (w/v) skim milk (Diploma) for 1 hour at room temperature with gentle rocking, or overnight at 4°C. The blocking buffer was then poured off and the primary antibody solution applied for 1 hour at room temperature. Mouse anti-his (Serotec), diluted 1:2,000 in blocking buffer was used as a positive control. When screening multiple samples, a Bio-Rad Mini-PROTEAN II multiscreen apparatus was used and samples added to individual lanes. The membrane was then washed three times in TBST, for 5 minutes each wash at room temperature, with gentle rocking. A solution of alkaline phosphatase conjugated goat anti-mouse IgG (Sigma), diluted 1:5,000 in blocking buffer was then added and the membrane incubated for 1 hour at room temperature with gentle rocking. The membrane was then washed as before. Western Blue colour development solution (Promega) was applied and the colour allowed to develop at room temperature. Colour development was stopped by rinsing the membrane in excess deionised water and the membrane allowed to dry.

5.2.3.3 Haemagglutination assay and correlation of protein amount to HA titre
A modified haemagglutination assay was performed as described by Raidal et al. (1993c), using both serial and non-serial dilutions of a 360 ng/µL stock solution of purified recombinant protein. The negative control consisted of PBS only and the positive control was a 50 µL suspension of virus purified from feathers and organs of a BFDV-infected sulphur-crested cockatoo, diluted to give a HA titre of 4-8 HAU/50 µL. The amount of protein in the first well of each row was calculated using the
dilution factor and HA titres were plotted against the calculated protein amounts (Figure 5.2).

5.2.3.4 Haemagglutination-inhibition assay
Haemagglutination-inhibition assays were carried out as described in Section 4.2.6, except that recombinant protein, diluted to 4-8 HAU/50 µL, was used as antigen.

5.2.3.5 Transmission electron microscopy
An aliquot of purified and concentrated recombinant protein solution was spotted onto copper 400 mesh Formvar carbon-coated grids and left to stand for 1 min at room temperature. The protein solution was then blotted off with Whatmann filter paper and the sample stained with phosphotungstic acid (PTA) by adding a drop of PTA and leaving the grid to stand for 1 minute at room temperature. The PTA was then blotted off and the grid viewed in a Philips CM100 transmission electron microscope.

5.2.3.6 Protein sequencing
Protein sequencing was carried out by Proteomics International (Perth, Western Australia). After SDS-PAGE analysis, samples of protein were cut out of the gels and extracted by trypsin digestion (Casey et al., 2005) followed by MALDI-TOF mass spectrometry using a 4800 Proteomics Analyser (Applied Biosystems). Spectra were analysed using Mascot sequence matching software (Matrix Science) and compared to entries in the NCBI MSDB database.
5.2.4 Production of monoclonal antibodies

5.2.4.1 Immunisation and screening of mice
A mixture of 50 µg of protein mixed with Freund’s Incomplete adjuvant was injected into the peritoneal cavity of each of 4 BALB/c mice. 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 and western blot as described above and by ELISA. 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.

5.2.4.2 Indirect enzyme-linked immunosorbent assay
The recombinant protein was diluted to 5 µg/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.6 and added to all wells of a 96-well ELISA plate (Costar), then the plate incubated at 4°C overnight. The plate was then washed 3 times with PBST plus 0.05% Tween 20 (PBST). A blocking solution, 5% BSA in PBST, was added to all wells and the plate incubated for 1 hour at room temperature. The plate was washed again and 50 µL of 5% BSA in PBS (PBS-BSA) added to all wells. When screening mice vaccinated with the recombinant protein, 50 µL of serum, diluted 1:100 in PBS-BSA, were added to the first well of each row and serially diluted across the plate. The plate was then incubated at room temperature for 1 hour, after which the sera were removed. The plate was washed 3 times as before, then 50 µL of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma), diluted 1:5, 000 in PBS-BSA were added to all wells and the plate incubated for 1 hour at room temperature. The solution was removed and the plate washed as before, with a final wash with deionised water. Colour development was carried out by adding a developing solution containing 2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)
and 10% (v/v) citric acid pH 4.2, then incubating at room temperature for 15 minutes. Absorbances were read at 405 nm using a DTX 880 spectrophotometer (Beckman Coulter).

Hybridoma supernatants were screened as above, except that 50 µL of hybridoma supernatant were added to the corresponding individual well of the ELISA plate. On a separate ELISA plate, known positive mouse serum (processed as above) was used as a positive control and negative mouse serum and PBS were used as negative controls.

5.2.4.3 Production and screening of hybridomas
Sp2/0 mouse myeloma cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 1% foetal calf serum (FCS) and antibiotics (penicillin and streptomycin), at 37°C in a 5% CO₂-in-air environment. Cell culture medium was changed every 2-3 days and cells were split between 1:2 to 1:5 (depending on the rate of growth) once they were 75% confluent or more.

The spleen from mouse BB01 was harvested aseptically, the connective tissue removed with forceps, the pulp passed through a fine mesh filter and the cells collected into DMEM. The cell suspension was then centrifuged at 1, 500 rpm for 5 minutes and the media decanted. Sp2/0 myeloma cells were also harvested. The spleen cells were resuspended in a small volume of DMEM and mixed with the myeloma cells. More DMEM was added to the cell suspension, the cells pelleted by centrifugation and the media decanted. One millilitre of PEG 1500 was then added slowly to the cells with gentle mixing, then 2 mL DMEM was added slowly with gentle
mixing. The cell suspension was then divided into two equal volumes and DMEM added to both aliquots, to a final volume of 40 mL. 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 serum-free medium (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 the hybridoma supernatants were screened by ELISA.

ELISA-positive hybridomas were transferred to a 48-well culture plate and 1 mL of Hybridoma-SFM (plus FCS, antibiotics and IL-6) added. The hybridomas were placed in an incubator and allowed to grow overnight, then re-screened by ELISA the following day to confirm that hybridomas were continuing to produce the desired antibodies. 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, using the supernatant of the hybridoma culture media. 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. The cells of the
selected hybridoma were resuspended gently in situ and a small aliquot taken to perform a viable cell count. The cells were then diluted to a final concentration of 45 viable cells per 10 mL culture medium and 100 µL of the cell suspension were added to all wells of a 96-well culture plate. 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.

Electrophoretic mobility shift assays were also conducted in an attempt to determine the affinity of the antibodies for the NLS. Initial assays were conducted as described by Heath et al. (2006), using polyclonal chicken anti-BFDV in order to assess the suitability of the assay for screening monoclonal antibodies. A total of 10 ng recombinant capsid protein was mixed in DNA binding buffer (100 mM Tris-HCl, pH 8, 300 mM KCl, 25 mM MgCl₂, 20% glycerol, 500 µg/ml BSA) in a 0.5 mL microcentrifuge tube (Eppendorf) to a volume of 20 µL. To this, 25 µL of chicken anti-BFDV polyclonal antisera, with an HI titre of 4-8 HIU/50 µL, were added and the mixture incubated for 1 hour at 37°C. One hundred nanograms of pUC 18 plasmid DNA (Invitrogen) were then added to make a total volume of 50 µL and the solution incubated again for 1 hour at 37°C. Two control reactions, one without protein and another without protein or antibodies were prepared in separate microcentrifuge tubes. Reaction products were visualised on a 1% agarose gel with the addition of 0.001% ethidium bromide, run at 90 V for 30 minutes. The polyclonal antibody solution was also purified by ammonium sulphate precipitation as described by
Nilson et al. (1993). Briefly, 100 µL of saturated ammonium chloride solution was added to an equal volume of chicken anti-BFDV polyclonal antisera and the solution incubated at 4ºC for 18 hours. The solution was then centrifuged at 10,000 rpm for 15 minutes, the supernatant removed and the pellet resuspended in 100 µL PBS. The purified antibodies were tested by HI assay to confirm the success of the purification, then used in another electrophoretic mobility shift assay.

5.2.4.4 Production and purification of monoclonal antibodies
The selected hybridoma was grown in Hybridoma-SFM (Gibco) plus FCS, antibiotics and IL-6 and expanded to a total volume of one litre. Before purification of the monoclonal antibody, hybridoma cells were pelleted by centrifugation at 1,500 rpm 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 50 mM glycine buffer (pH 2.7), into Tris buffer (1 M Tris-HCl, pH 8). Purified antibodies were then dialysed in PBS overnight at 4ºC. The subclass of the antibody was determined using a Pierce mouse isotyping kit (Pierce), with the assay performed in triplicate.

5.2.5 Applications of the monoclonal antibody
5.2.5.1 Haemagglutination inhibition
Fifty microlitres of undiluted purified monoclonal antibody were tested by HI as described above, without adsorption against either kaolin or normal galah erythrocytes.
5.2.5.2 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 2.5 µg/mL in 0.05 M 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 were 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) were 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.

5.2.5.3 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 multiscree apparatus and incubated at room temperature for 1 hour. Colour development was carried out as previously described.

5.2.5.4 Optimisation of indirect immunohistochemistry
Three different dilutions of the monoclonal antibody (1:50, 1:200 and 1:500) were used in an indirect IHC procedure to optimise the amount of antibody. Five-micron sections of formalin-fixed and paraffin embedded liver tissue, from a rainbow lorikeet 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 minutes and re-hydrated using decreasing ethanol concentrations and a final wash in Tris buffer for 3 minutes. 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 and then the sections were 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. Antigen-antibody complexes were visualised by the addition of the chromogen diaminobenzidine (DAKO® DAB chromogen) 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. Imaging was performed using an Olympus BX 13
microscope and digital camera accessory.

5.2.6 Development of a blocking enzyme-linked immunosorbent assay
Insofar as was practical, the bELISA was developed and validated in accordance
with guidelines established by the Office International des Epizooties (O.I.E.) for the
development of serological assays for the diagnosis of infectious diseases
(Jacobson, 2004).

5.2.6.1 Samples
Samples of blood, collected on filter paper and air-dried and serum or plasma from 5
species of psittacine birds (including sulphur-crested cockatoos, galahs, rainbow
lorikeets, eclectus parrots and gang-gang cockatoos known to have antibodies
against BFDV were used to optimise the Ab-bELISA. These were selected to include
a range of HI antibody titres. Normal chicken serum was included as a known
negative control and a sample of serum from a chicken inoculated with a DNA
vaccine protective for BFDV (Section 6.2.6) was included as a sample which had an
antibody titre below the limit of detection of the HI assay.

To validate the bELISA, 166 samples from the vaccine trial described in Section
3.2.3 were tested using the optimised bELISA. 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 (PBS, 0.05% (v/v) Tween 20, 5% (w/v) skim milk powder (Diploma)) were 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.

Feathers from birds which had previously tested positive for BFDV by haemagglutination assay were used in the development of the antigen blocking ELISA (Ag-bELISA). Samples were selected to include a range of HA antigen titres and were prepared as for haemagglutination assays as described by Raidal et al. (1993c). Feathers were incubated with 100 µL PBS at 60ºC for 1 hour in a microcentrifuge tube (Eppendorf) and the suspension centrifuged briefly.

5.2.6.2 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 were added to all wells of the ELISA plate and 50 µL sera, eluted as above, were 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 (Section 5.2.4), diluted 1:400 in blocking buffer, were 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, were 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) were 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 405 nm 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.

5.2.6.3 Optimisation of the blocking enzyme-linked immunosorbent assay for the detection of antigen (antigen-bELISA)
Microtitre plates were coated overnight with recombinant protein, washed, and blocked as described above. At the same time, 50 µL blocking buffer were added to a separate, non-coated microtitre plate and 50 µL of the supernatants from individual feather preparation were added to the first well of each column. Serial dilutions were made by removing 50 µL of the solution from the first well, mixing with 50 µL PBS in the next well and repeating the process down the column of the microtitre plate. Fifty microlitres of the monoclonal antibody, diluted 1:400 in blocking buffer, were added to each well. Negative and positive controls lanes were included for each microtitre plate; negative (no inhibition) controls used blocking buffer instead of the feather preparation and positive (complete inhibition) controls used blocking buffer instead of the antibody solution. The plates were then incubated as described above, then the protein-coated plate washed and the feather eluate/antibody mixture transferred from
the non-coated plate to the coated plate. The plate was incubated and washed, then colour development and calculation of PI values carried out as described above.

Further optimisation was conducted by testing the monoclonal antibody at dilutions of 1:400, 1:1,500, 1:3,000, 1:5,000, 1:10,000, 1:20,000 in blocking buffer and using serial dilutions of a 10% feather suspension from sulphur-crested cockatoo with a strong positive HA titre of >40 960HAU/50 µL. Each antibody dilution was tested in parallel using blocking buffer with 5% skim milk, blocking buffer with 1% skim milk and plain PBS as the diluent.

**5.2.7 Validation of the Ab-bELISA for the detection of specific antibodies**

To validate the bELISA, 166 samples from eastern long-billed corellas vaccinated with a recombinant BFDV capsid protein (Bonne et al., 2008b, Stewart et al., 2007) and 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 (Section 4.3.1) as well as samples from a cockatiel known to be BFDV-positive and its cage mate were also tested. Both of these birds had also previously tested negative by HI. For blood collected onto filter paper one spot of blood on filter paper was cut out using scissors or a hole punch and collected into a microcentrifuge tube (Eppendorf). 100 µL of ELISA blocking buffer were 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 test of intra-assay repeatability was plotted to determine the assay’s precision. The linearity of the assay was also investigated using 10 replicates of the pooled sera that were serially diluted 1:2 and the PI values plotted against the log₂ of the dilution factor.

5.2.8 Statistical analysis
Calculation of the line of best fit and correlation coefficient of the graph of protein amount against HA titre, the PI values of vaccinated and non-vaccinated birds against time, the correlation between HI values and bELISA PI values were performed using CoPlot (CoHort Software, 2005). Descriptive statistics for bELISA PI values from the vaccine trial, sensitivity, specificity, positive and negative predictive values and coefficients of variation were calculated using Microsoft Excel.

5.3 Results
5.3.1 The recombinant protein has HA activity and forms virus-like particles
The recombinant protein was successfully expressed in Sf9 insect cells and was purified using both Ni-NTA resin chromatography and sucrose/CsCl ultracentrifugation. Purification using Ni-NTA resin was incomplete, however, with much of the protein remaining in the insoluble and unbound fractions (Figure 5.1a). Sucrose/CsCl ultracentrifugation successfully purified the protein from both of these
fractions and was also used successfully to purify the protein from crude insect cell lysates (Figure 5.1b). Dialysis in PEG 20K could successfully concentrate the protein more than 100-fold overnight.

SDS-PAGE showed that the protein was approximately 32 kDa in size (Figure 5.1a) and was not present in the control Sf9 cells (not shown). Western blotting with mouse anti-his antibodies detected only this 32 kDa protein.

![Image of SDS-PAGE and Sucrose/CsCl purification](image)

Figure 5.1: Purification of the recombinant protein by Nickel-NTA resin and caesium chloride (CsCl) ultracentrifugation. A: SDS-PAGE analysis of the expressed and purified protein (arrow). MW: molecular weight markers; Ins: the insoluble proteins, collected after clarification of the lysed cells; Unb: the unbound fraction (proteins which did not bind to the Ni-NTA resin); W1 – 3: wash fractions 1 to 3; E1 – 4: elution fractions 1 to 4. Note that the protein is present in the first elution fraction, as it should be, but a large amount of protein is still present in both the insoluble and unbound fractions. B: Sucrose/CsCl purification of the recombinant protein. A distinct band containing the protein can be seen (open arrow).

The recombinant protein demonstrated haemagglutination activity with Type A galah erythrocytes (Figure 5.2a, b) and protein concentration was logarithmically related to the HA titre ($\log_2 HA = 1.65\ln(\text{protein}) + 2.25$, $R^2 = 0.952$, $p<0.05$) (Figure 5.2c).
Figure 5.2: Correlation of protein amount with haemagglutination (HA) titre. A: Serial dilutions of the recombinant protein. B: non-serial dilutions of the recombinant protein. C: Relationship between protein amount (ng/well) and log₂HA titre.

Sera from naturally immune psittacine birds successfully inhibited agglutination of galah erythrocytes, as did the polyclonal chicken anti-BFDV used as the positive control. The expected HI titre of each bird’s serum was achieved for all samples. Sera from naïve chickens and psittacine birds did not inhibit agglutination by the recombinant protein (Figure 5.3).
Figure 5.3: Haemagglutination inhibition (HI) assay using the recombinant protein as the antigen source and sera from inoculated chickens and naturally immune psittacine birds. The first row is a back titration of native Beak and Feather Disease Virus and the second row a back titration of the recombinant protein. The sera from naturally immune psittacine birds (rows 3-8) attained the expected HI titres. A positive HI titre was indicated by the sedimentation of the erythrocytes within the well of the microtitre plate, whereas a negative HI result was indicated by the suspension of the erythrocytes in the well.

Transmission electron microscopy showed that the protein self-assembled into spherical, 18-20 nm diameter VLPs that appeared identical to wild-type BFDV virions (Figure 5.4). Protein sequencing by MALDI-TOF mass spectrometry showed the recombinant protein to be similar to putative BFDV capsid protein sequences.
5.3.2 A monoclonal antibody to a recombinant BFDV capsid protein
All mice developed an antibody response to the recombinant protein detectable by western blotting and the polyclonal anti-recombinant BFDV Cap serum from all mice recognised native BFDV in an HI assay (Figure 5.5b). The spleen cells from mouse BB1 were fused with Sp2/0 myeloma cells and about 60% of the resultant hybridomas grew very well.
ELISA assays showed that most (>70%) hybridomas were producing antibody that recognised the recombinant protein (Figure 5.6b), 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 (i.e. haemadsorption only or no treatment), non-specific HA activity was present in the samples (Figure 5.6c). Haemadsorption prior to testing reduced this non-specific HA activity by a factor of $\log_2 2$ and both kaolin and haemadsorption eliminated non-specific HA (Figure 5.6d).

Electrophoretic mobility shift assay revealed that proteins in the antiserum solution also bind DNA. The control assay containing antiserum but not protein inhibited DNA movement as much as the assay containing protein only. The antiserum was successfully purified by ammonium sulphate precipitation, as the purified solution
had the same HI titre as the non-purified solution. The control assay containing purified antibodies but not protein did not inhibit DNA movement. However, the test assay containing protein and purified antibodies did not prevent the inhibition of DNA movement by the recombinant protein. Thus it was concluded that larger quantities of antibody than were available are likely required to inhibit the binding of the recombinant protein to DNA.

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 5.7a). 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 (Figure 5.7a, arrow). Hybridoma 3F8 was cloned by limiting dilution and screened by both ELISA and western blot. Three clones were determined to be positive by ELISA and all clones detected only the recombinant protein and not the PCV2 capsid protein or polyhistidine tag by western blot (Figure 5.7b). Clone 3F8-1 had the strongest reaction by ELISA and western blot and was chosen for expansion and production of monoclonal antibodies. After purification, the subclass of the monoclonal antibody was determined to be IgG2a, with a kappa light chain.

Figure 5.7: A: Western blotting of IgG-secreting, ELISA-positive hybridomas. Hybridoma 3F8 (arrow) reacted strongly with the recombinant protein. M: molecular weight markers; Lane 1: polyclonal mouse anti-recombinant Beak and Feather Disease Virus Cap; Lanes 2-14: tested hybridoma supernatants. Note hybridoma 3F8 (lane 5, arrow). B: western blotting of limiting-dilution clones of hybridoma 3F8 against the recombinant BFDV capsid protein (lanes 1-4) and a his-tagged recombinant Porcine Circovirus 2 capsid protein (lanes 5-8). M: molecular weight markers; Lanes 1, 5: monoclonal mouse anti-his; Lanes 2-4, 6-8 clones 3F8-1 to 3F8-3.
5.3.3 The monoclonal antibody has HI activity and is useful in multiple applications

5.3.3.1 Haemagglutination inhibition
Hybridoma 3F8-1, when untreated by either kaolin or haemadsorption, had an HI titre of between \( \log_2 2 \)-\( \log_2 4 \), depending on the amount of virus used in the test (Figure 5.8).

![Figure 5.8: Haemagglutination Inhibition (HI) assay using monoclonal antibody 3F8-1. When the virus was used at low concentrations, the monoclonal antibody had HI activity of up to \( \log_2 2 \) HIU/50µL. Row 1: a back titration of Beak and Feather Disease Virus (BFDV) (open arrow); Row 2: polyclonal chicken anti-BFDV; Row 3: monoclonal antibody 3F8-1 used as a test sample (closed arrow).]

5.3.3.2 Indirect enzyme-linked immunosorbent assay
The optimal amount of protein used in the indirect ELISA was determined to be 250 ng/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.

5.3.3.3 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 1:4,000 (Figure 5.9).
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 (Figure 5.10b, d) 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 5.10c, e, g).
Figure 5.10: Indirect immunohistochemistry using monoclonal antibody 3F8-1 on a section of liver from a lorikeet infected with Beak and Feather Disease Virus. A: negative (no monoclonal antibody) control; B, D, F: the monoclonal antibody used at 1:50, 1:200 and 1:500, respectively, without antigen retrieval; C, E, G: the monoclonal antibody used at the same respective dilutions, with antigen retrieval. Note the increased edge-effect around erythrocytes with the higher antibody concentration (image B) and the decrease in endogenous peroxidase activity, background staining but also specific staining after antigen retrieval (image C, E, G). Bar equals 50 µm.
5.3.4 A novel blocking ELISA for the detection of antibodies to BFDV

The optimal dilution of serum for use in the Ab-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.

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 (Figure 5.11).

![Figure 5.11: Blocking ELISA (bELISA) mean percentage inhibition values of vaccinated (○) and control (●) birds after vaccination. Vaccinations are indicated by an arrow (↑) and challenge with a triangle (△). Bars indicate standard error.](image-url)
Control birds had peak antibody levels of between 55.25% and 83.26% after challenge with live virus. When antibody levels measured by bELISA were compared with HI titres, the correlation coefficient was very high ($r^2 = 0.8156$, $p<0.05$) (Figure 5.12). After repeating the cockatiel sero-survey using the Ab-bELISA, no antibodies were detected in the serum of any of the birds. Furthermore, both the cockatiel known to be BFDV-positive and its cage-mate were also negative by bELISA.

![Figure 5.12: Correlation of Haemagglutination Inhibition titres with blocking ELISA percentage inhibition.](image)

Based on the results of the vaccine trial samples, 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 (Figure 5.13a). 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 5.13b). This working range did not include the entire biological range exhibited by samples from birds in the vaccine trial, however.

Figure 5.13: A: Blocking ELISA (bELISA) precision. B: bELISA linearity assay using sera pooled from 10 vaccinated birds. The percentage inhibition values are linear within the dynamic range of the test. Bars indicate standard error.

The Ag-bELISA was not useful for the detection of BFDV antigen, since the binding of the monoclonal antibody to the recombinant protein on the microtitre plate was not inhibited by naturally occurring amounts of soluble antigen at a dilution of 1:10,000 or less. At dilutions greater than 1:10,000, spectrophotometer measurements were subject to increased analytical error and were not appropriately accurate or repeatable.
5.4 Discussion
Given the high rates of BFDV infection in psittacine birds worldwide, sensitive and specific detection methods which are readily standardised, making comparison of results between different laboratories feasible, 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 in this study also has the advantage that it can 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.

Like other recombinant viral proteins (Noad and Roy, 2003, Tegerstedt et al., 2005, Ulrich et al., 1998), the full-length recombinant BFDV capsid protein self assembled into VLPs. Importantly, the VLPs behaved like native BFDV in that they had HA activity and were recognised by naturally occurring anti-BFDV sera. Other recombinant BFDV capsid proteins have been developed (Heath et al., 2006, Johne et al., 2004) and have been shown to have merit for use in serological assays, but the morphology and haemagglutination behaviour of the proteins were not assessed. The formation of VLPs is an important consideration in the development of vaccines for BFDV, as VLPs are processed in a similar way to native virus (Noad and Roy, 2003, Tegerstedt et al., 2005, Ulrich et al., 1998) and induce potent T-cell responses (Noad and Roy, 2003). The variable size and shape of some of the VLPs is probably
a result of incomplete or improper assembly (Crowther et al., 2003, Wong et al., 1994). A criticism of HA and HI tests is that the variation in sensitivities of donor erythrocytes to the virus, which may induce differences in results when the same amount of virus or antibody is used, makes them difficult to standardise. The experiment performed in this chapter to correlate the amount of protein with the resultant HA titre allows a measure of standardisation of HI assays as well as a means of assessing the efficiency of the various steps of the purification process. Obviously the HA titre of a protein sample will differ when erythrocytes from different hosts are used. The sensitivity of a single donor’s erythrocytes varies over time, but the precise measurement of protein quantity allows this variation to be accounted for and mitigated to a certain extent.

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, the current 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 amount of virus that the authors used may have affected the inhibition of haemagglutination by the monoclonal antibody, as the HI assay was described as using virus with an HA titre of 64 HAU (Ritchie et al., 1991b). Why the authors used this amount of virus is unclear. 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 monoclonal antibody detected virus from a cockatoo and a lorikeet (this study) and a cockatiel (Section 4.3.2). 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, the results of the similar experiment described in Section 4.3.1 suggest that the cockatiel isolate may be sufficiently different antigenically to be considered a separate serotype. 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 potentially has 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, presented in the current study, are promising and the antibody should have widespread diagnostic application after its efficacy has been tested with a suitable range of virus isolates.

At present, HI and HA assays are the best available assays for the detection of anti-BFDV antibodies and shed virus, respectively. However, given the amount of variation inherent in both the HI and HA assays, novel diagnostic tests for both the virus and the presence of anti-BFDV antibodies in serum are highly desirable. 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 bELISAs 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 sensitivity and specificity of the blocking ELISA developed here were within values defined as acceptable by the OIE (Jacobson, 2004) and should prove to be a useful diagnostic test for BFDV. The monoclonal antibody used in the assay has a
high analytical specificity (Section 5.3.2) and the analytical sensitivity was greater
than the HI test, as it detected antibodies in vaccinated birds that were HI negative.
Because of this, however, the analytical sensitivity could not be determined
precisely. The comparative diagnostic sensitivity and specificity was good, but
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 were within values accepted
by the OIE (Jacobson, 1998). 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, the 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 correlation coefficient between bELISA values and HI titres from a group of corellas vaccinated with a recombinant BFDV capsid protein and challenged with live virus (Bonne et al., 2008b) was high. This 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, 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, but further testing and validation should be an ongoing process. The fact that the monoclonal antibody described in this chapter recognised BFDV from a sulphur-crested cockatoo, rainbow lorikeet and a cockatiel indicates that there is some antigenic homology between isolates.

The sero-survey of 88 cockatiels described in Chapter 4 failed to find any birds with detectable HI titres. As such, the sero-survey was repeated using the newly developed bELISA and included serum from a cockatiel known to be BFDV-positive and its cage mate in an attempt to elucidate the reason for the apparent universal negative results. The apparent lack of sero-positive birds may be due to one of three possibilities. Firstly, the birds may have had antibody levels below the limit of detection of the HI test. Secondly, antibodies in cockatiel sera may not cross-react with the virus used in the HI tests or the recombinant protein, or finally, cockatiels may be resistant to BFDV infection to the extent that they rarely become infected and hence do not develop antibodies. Unfortunately, no definite answer can be deduced after repeating all of these samples, given 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. A definitive answer to this problem may only be discovered by conducting HI assays using sera from many cockatiels, tested against virus eluted from the feathers from a range of psittacine birds.

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 more 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.

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.

It is unfortunate that the bELISA could not be adapted for the detection of antigen as well. Since the monoclonal antibody has been shown to bind to soluble antigen in solution in an HI assay, the most likely explanation for the lack of success of the Ag-bELISA is that the dilution of monoclonal antibody required for it to be the limiting reagent was too great. A 1:400 dilution was appropriate for the Ab-bELISA, since the amount of recombinant protein was the limiting factor and a greater amount of monoclonal antibody could be used in order to amplify the signal. It is unlikely that the Ag-bELISA was not successful due to the monoclonal antibody having a greater affinity for the recombinant protein than native virus, since it has been shown that the optimal dilution of monoclonal antibody is the same for recombinant protein, in western blotting and for virus in IHC.

An antigen-capture ELISA will almost certainly be more useful for the antigen detection and such an assay could easily be developed using the components of the Ab-bELISA. In this assay, the monoclonal antibody would be bound to the microtitre plate, virus suspensions added and then the same monoclonal antibody, directly conjugated to an enzyme such as HRP bound to the captured virus and colour developed and absorbances measured. Alternately, given the large number of hybridomas available for development, a second monoclonal antibody could be produced and used in the capture ELISA, with the antibody of greatest affinity bound to the microtitre plate. If epitope mapping experiments are carried out with different BFDV isolates and antigenic differences are revealed, antigen capture ELISAs could potentially be developed to discriminate between isolates.
Chapter 6. Development of Two Novel DNA Vaccines as a Potential Strategy for the Control of Psittacine Beak and Feather Disease (PBFD)

6.1 Introduction and Aims

6.1.1 Introduction
Psittacine Beak and Feather Disease (PBFD) is the most common viral infection of psittacine birds and causes both a chronic debilitating feather disease in adult birds (Albertyn et al., 2004, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989b) and a severe, acute disease syndrome in nestlings and African grey parrots (Doneley, 2003, Raidal and Cross, 1995, Schoemaker et al., 2000). The causative agent, BFDV, is a circovirus with a single stranded DNA genome, approximately 1.7-2.0 kb in length (Bassami et al., 2001, Ritchie et al., 1989b). The genome of BFDV and other circoviruses is ambisense and encodes at least two major open reading frames (ORFs) (Bassami et al., 1998, Hattermann et al., 2003, Khalesi et al., 2005, Todd et al., 2001a, b). The ORF present on the viral strand of the double-stranded DNA replicative form (Hanley-Bowdoin et al., 1999) encodes the replication-associated protein, responsible for initiation of rolling circle replication (Hanley-Bowdoin et al., 1999, Steinfeldt et al., 2001, Steinfeldt et al., 2006). The ORF present on the complementary strand encodes the capsid protein and studies using recombinant proteins have demonstrated that Cap is the major antigenic determinant of BFDV (Stewart et al., 2007).

As no cell culture system has been developed to successfully grow the virus in vitro, recombinant techniques hold the most promise for the development of effective vaccines that may be produced on a large scale (Bonne, 2004, Heath et al., 2006,
A killed whole-virus vaccine has been developed previously (Raidal and Cross, 1994a, Raidal et al., 1993a), but the antigen was purified from the feathers of 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. Incomplete inactivation of the virus may also result in clinical disease when administered (Raidal et al., 1993a, Wylie and Pass, 1987).

DNA vaccines have been developed against many aetiological agents including viruses (Cheung et al., 2004, Li et al., 2004, Wang et al., 2006, Yang et al., 2004), bacteria (Ko et al., 2005) and parasites (Lillehoj et al., 2005, Nagata et al., 1999, Wu et al., 2004). In order to explore the potential of DNA vaccines for the control of BFDV, two similar vaccines based on the nucleotide sequence of the capsid-coding gene of BFDV were constructed and their expression both in vitro and in vivo and the subsequent antibody response incited in the latter were examined. To test the hypothesis that removal of the NLS would result in improved expression (Heath et al., 2006) and consequently an increased host immune response, DNA vaccines encoding both the full-length and NLS-truncated capsid sequence were assessed.

6.1.2 Aims
The principal aim of this experiment was to determine whether a DNA vaccine based on the capsid sequence of BFDV would incite the development of antibodies in vaccinated birds. Secondary aims were to determine whether there was a difference in antibody levels between groups of birds administered either the full-length or truncated vaccine and whether peak antibody levels might be protective against infection with BFDV.
6.2 Materials and Methods

6.2.1 Experimental design
Vaccine type (full-length or truncated) was the independent variable when assessing the performance of the vaccines both in vitro and in vivo. When assessing in vitro performance, the dependent variable was the subcellular location of the expressed proteins. When assessing in vivo performance, the dependent variable was the antibody level in vaccinated birds, assessed by bELISA. The vaccination experiment consisted of two treatments (vaccine type), with three replicates per treatment group and was a balanced design. Each treatment was performed twice and samples were collected before treatment, at the time of each treatment and 3 times after the second treatment.

6.2.2 Insert generation by PCR
Viral DNA was extracted from the feather of a sulphur-crested cockatoo which had previously tested PCR positive for BFDV using the methods described by Ypelaar et al. (1999) (Section 3.2.4).

Primers CapFullF1 and CapR (Table 6.1) were designed to generate a PCR product coding for the full-length 747 nt capsid-coding region of BFDV; primers Cap-nlsF3 (Table 6.1) and CapR were used to generate an NLS-truncated variant, which was without the first 168 nt at the 5’ end (bases in red type indicate a Kozac sequence).
Table 6.1: Primers used for the amplification and screening of capsid-coding inserts for use in the DNA vaccine

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CapFullF1</td>
<td>5’ GAT TAT GGG AGG CAC CTC CAA C 3’</td>
<td>1961-1975 (after Kozac)</td>
</tr>
<tr>
<td>Cap-nlsF3</td>
<td>5’ GAC CAT GGG CCA ATT CAA ATT C 3’</td>
<td>1798-1812 (after Kozac)</td>
</tr>
<tr>
<td>CapR</td>
<td>5’ TTA AGT GCT GGG ATT GTT AGG GGC 3’</td>
<td>1235-1257</td>
</tr>
<tr>
<td>T7 forward</td>
<td>5’ TAA TAC GAC TCA CTA TAG GG 3’</td>
<td></td>
</tr>
</tbody>
</table>

*Type in red indicates a Kozac sequence

PCR reactions consisted of 2.5 mM MgCl$_2$, 2.5 µL of 10x polymerisation buffer, 25 pmol of each primer, 0.1 mM of each dNTP, 0.12 U of Taq DNA polymerase plus ultrapure water to a final volume of 23 µL (all reagents Fischer Biotec). Two microlitres of DNA were added and reactions carried out in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf). Cycling conditions are outlined in Table 6.2. After the PCR reaction, a small aliquot of the PCR products was loaded on a 1% agarose gel containing ethidium bromide, subjected to electrophoresis at 90 V for 1 hour and viewed on a transilluminator. The rest of the PCR product was purified using an Axyprep PCR cleanup kit (Axygen), according to the manufacturer’s instructions.

Table 6.2: Cycling conditions used for the amplification of capsid-coding inserts for use in the DNA vaccine

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (ºC)</th>
<th>Time (s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
6.2.3 Construction and production of plasmids pVAX1BFDVC1 and pVAX1BFDVC1-nls

Plasmid pVAX1 (Invitrogen) was subjected to restriction digestion with EcoRV, to give blunt ends. The reaction mixture consisted of 1 µg of pVAX1, 3 µL of EcoRV 10x reaction buffer, 3 µg BSA and 5 U EcoRV, plus sterile deionised water to a total volume of 25 µL (all reagents Fischer Biotec). The reaction was incubated for at least 1 hour at 37°C, then purified using an Axyprep PCR product purification kit.

After purification, 5’ T-overhangs were added by incubating the digested and purified plasmid with 2.5 mM MgCl$_2$, 5 U of Taq DNA polymerase, 10 µL of 10x Taq amplification buffer, 1 mM dTTP and ultrapure water to a total volume of 100 µL. The reaction mixture was incubated for 2 hours at 75°C, then purified using an Axyprep PCR product purification kit.

Before the ligation reaction was performed, the amounts of vector and insert DNA were measured using a Nanodrop ND-100 spectrophotometer. The volumes of vector and insert DNA required was calculated using the formula:

$$x \text{ (ng PCR product)} = \frac{(Y \text{ bp PCR product})(50 \text{ ng pVAX1})}{(\text{size in bp of pVAX1: } \sim 3,000 \text{ bp})}$$

The vector and insert DNA were then mixed together along with 1 µL of 10x ligation buffer and 1 µL of T4 DNA ligase (all reagents Invitrogen), then made up to 10 µL with ultrapure water and incubated at 14°C overnight.
Chemically competent TOP10F’ *E. coli* (Invitrogen) were transformed with the plasmids pVAX1BFDVC1 and pVAX1BFDVC1-nls. Two microlitres of each transformation reaction were added to 50 µL of competent cells and the mixture incubated on ice for 30 minutes. The cells were then heat-shocked by incubation at 42°C for 30 seconds in a water bath and then placed on ice. Two hundred and fifty microlitres of SOC medium (2.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$.6H$_2$O, 20 mM glucose; Invitrogen) were added and the mixture incubated for 1 hour at 37°C on a horizontal shaker (Certomat$^\text{®}$; R.B. Braun) at 225 rpm. Aliquots of 25 and 50 µL of each transformation mixture were plated onto 2xYT agar plates (16 g/L tryptone peptone, 10 g/L yeast extract, 5 g/L NaCl, 15 g/L agar (Becton Dickinson) 50 µg/mL kanamycin). Plates were incubated at 37°C overnight.

### 6.2.4 Screening of *E. coli* and plasmids for the inserted capsid sequence

#### 6.2.4.1 Growth of selected colonies
Colonies of cells transformed with both pVAX1BFDVC1 and pVAX1BFDVC1-nls were screened in the same way. Fifteen colonies each were selected and each colony inoculated both onto a new 2xYT agar plate marked with a grid and into 10 mL of 2xYT broth (16 g/L tryptone peptone, 10 g/L yeast extract, 5 g/L NaCl, Becton Dickinson) with 50 µg/mL kanamycin added. The inocula on plates were incubated overnight at 37°C. Broth was likewise incubated, but on a horizontal shaker as described above. The following morning, plates were wrapped in clear plastic film and stored at 4°C and colonies in broth were centrifuged at 14,000 rpm to pellet the cells, then stored at -80°C.
6.2.4.2 PCR screening of the selected colonies
Pelleted cells were screened by PCR using a toothpick miniprep procedure (Maniatis et al., 1982). A small amount of cells was picked up with a wire loop and dipped in ultrapure water (Fischer Biotech) and the suspension boiled for 10 minutes. Plasmid pVAX1 contains binding sites for the primer T7 forward within the T7 promoter region. Primers T7 forward (Table 6.1) and CapR were used to determine whether the capsid sequences were present and in the correct orientation, as only correctly oriented inserts would amplify.

PCR reactions consisted of 2.5 mM MgCl₂, 2.5 µL of 10x polymerisation buffer, 25 pmol of each primer, 0.1 mM of each dNTP, 0.12 U of Taq DNA polymerase plus ultrapure water to a final volume of 23 µL (all reagents Fischer Biotec). Two microlitres of the boiled toothpick miniprep were added and reactions carried out in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf). PCR and subsequent agarose gel electrophoresis was carried out as described above, except that the annealing temperature was decreased to 45°C.

6.2.4.3 Sequencing of PCR-positive colonies
Plasmid DNA was purified from the selected colonies using a HiPure Plasmid miniprep kit (Invitrogen). Dideoxynucleotide sequencing was carried out using primers T7 forward and CapR in an ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions, except that the reaction volume was reduced to 10 µL and the temperature reduced to 50°C. Sequencing reactions consisted of 2 µL of reaction buffer (containing Tris-HCl, MgCl₂, fluorescently-labelled dNTPs and AmpliTaq DNA polymerase, concentrations not supplied; Perkin Elmer), 1 µL of 5x sequencing buffer (composition not supplied,
Perkin Elmer), 150-200 ng of plasmid, then made up to a total of 10 µL with ultrapure water (Fischer Biotec).

DNA sequences were determined using an Applied Biosystems 3730 DNA Analyser and edited using Sequence Scanner v1.0 (Applied Biosystems) and GeneTool Lite (BTI Software). Edited sequences were analysed using MEGA 3.1 (Kumar et al., 2004). Inserts were sequenced at least twice in each direction and edited sequences compared to known BFDV capsid sequences using the programs BLASTn (Altschul et al., 1997) and MEGA 3.1 (Kumar et al., 2004).

6.2.5 Confirmation of protein expression in vitro

6.2.5.1 Cells and media
COS-7 cells (transformed African green monkey kidney cell line, ATCC CCL-1651) were grown in 25 cm³ tissue culture flasks (Falcon) in DMEM (Gibco) supplemented with 1% new born calf serum and antibiotics (penicillin and streptomycin), at 37°C in a 5% CO₂-in-air environment. Cell culture medium was changed every 2-3 days and cells were passaged once they were 75% or more confluent. When cells were passaged, the media was first aspirated then the cells washed with PBS. The PBS was then removed, 0.5 mL ATV (NaCl 80 g/L, KCl 4 g/L, glucose 10 g/L, NaHCO₃ 6 g/L, trypsin [1:250 of a 5 g/L solution], EDTA 2 g/L, 1% phenol red 4 mL/L) added and the cells incubated at 37°C for at least 5 mins until the cells had detached. Cells were resuspended in an appropriate volume of growth media and dispensed into cell culture flasks for further growth.
6.2.5.2 Transfection of COS-7 cells
The day before transfection, cells were split 1:5 into 6-well tissue culture flasks (Nunc) and grown overnight as above. The following day, 20 µg of each plasmid was mixed with 2 mL of calcium phosphate transfection buffer (0.25 M CaCl$_2$, 25 mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (Sigma), 140 mM NaCl, 0.75 M Na$_2$PO$_4$, pH 6.95) and 50 µL of the mixture added drop wise to the relevant wells of the culture flask. Transfection buffer without plasmid was also added to two wells as negative transfection controls. The culture plates were incubated overnight as above and the following day the cells were washed twice with fresh culture media, then fresh media was added once again and the cells incubated for 2 days as above. After this final incubation, the cells were washed twice with PBS then fixed in -20ºC methanol for 20 minutes.

6.2.5.3 Analysis of COS-7 cells for transient protein expression
Cells were assayed for transient protein expression via an indirect immunocytochemistry assay, using a monoclonal antibody (Section 5.3.2) developed against a recombinant BFDV capsid protein(Section 5.3.1). Fixed cells were washed twice with PBS, then a 1:500 dilution of the primary monoclonal antibody in PBS added and the cells incubated for 1 hour at room temperature. Unbound antibody was then removed by duplicate washes in PBS before incubating with HRP conjugated EnVision anti-mouse (Dako) at room temperature for 1 hour. The slide was washed as before and then antigen-antibody complexes visualised with the chromogen diaminobenzidine (DAKO® DAB chromogen). After colour development, cells were counterstained briefly with haematoxylin and imaging was performed using an Olympus BX 13 microscope and digital camera accessory.
6.2.6 Vaccination of chickens with plasmids pVAX1BFDVC1 and pVAX1BFDVC1-nls

6.2.6.1 Birds
Chickens were used as an experimental bird because the species is not known to be naturally susceptible to BFDV and this avoided any possibility of stimulating an anamnestic response. Six Isabrown chickens were housed at the Murdoch University Animal House, each of the two DNA vaccines was administered to each of three chickens. Five days before vaccination with the DNA vaccine plasmids, chickens were injected with 0.3 mL bupivicaine into the pectoral muscle to induce muscle regeneration. Chickens were injected twice with 300 µg of the DNA vaccine-containing plasmids at 14 day intervals and blood collected by venepuncture of jugular or cutaneous ulnar veins at each vaccination, then at fortnightly intervals after the second vaccination. Blood was spotted onto filter paper (Whatmann Grade No. 3), then allowed to air dry at room temperature as described by Riddoch et al. (1996). Serum from the chickens was tested for the presence of anti-BFDV antibodies by HI assay and the Ab-bELISA described in Section 5.2.6.2.

6.2.6.2 Haemagglutination-inhibition (HI) assay
Haemagglutination-inhibition assays were carried out as described in Section 4.2.6, except that the antigen preparation consisted of virus purified from the feathers of a sulphur-crested cockatoo with PBFD, diluted to give a HA titre of 4-8 HAU/50 µL.
6.2.6.3 Antibody-blocking ELISA
Antibody was also detected using a bELISA which utilised the recombinant baculovirus-expressed BFDV capsid protein-coated described in Section 5.3.1 and the BFDV-specific monoclonal antibody described in Section 5.3.2. The bELISA was performed as described in Section 5.2.6.2, with serum samples diluted 1:10 in blocking buffer.

6.2.7 Statistical analysis
Descriptive statistics and paired t-tests, between pre- and post-vaccination bELISA PI values, were performed using Microsoft Excel. Repeated measures ANOVA was performed using SPSS version 15.0.

6.3 Results
6.3.1 Generation of DNA vaccines for BFDV
Full-length and NLS-truncated capsid-coding sequences were successfully generated by PCR and cloned into vector pVAX1 (Invitrogen) to create plasmids pVAX1BFDVC1 and pVAX1BFDVC1-nls. PCR screening identified plasmids that contained the insert in the correct orientation (Figure 6.1) and sequencing of these products confirmed the inserts to be in the correct orientation and in-frame with the Kozac sequences and stop codons. These sequences had a high degree of homology with published BFDV sequences.
6.3.2 The DNA vaccines are successfully expressed *in vitro*

After indirect immunocytochemistry, both variants of the capsid protein were visible within transfected COS-7 cells as aggregates of brown pigment (Figure 6.2). The subcellular localisation of the protein within mammalian cells was in agreement with the findings of Heath et al. (2006), who examined the location of the expressed protein in insect cells. In COS-7 cells, the full-length capsid protein was present in the nuclei of transfected cells, whereas the NLS-truncated protein was present in the cytoplasm. Visualisation of the protein within nuclei was easier before counterstaining, after which cells expressing the full-length capsid protein were identified by the intense dark colour of the nucleus.
6.3.3 The DNA vaccines are expressed in vivo and vaccinated birds produce antibodies against BFDV

HI assays were confounded by the presence of non-specific agglutinins, present despite adsorption with kaolin and normal galah erythrocytes. Consequently, no results were obtained from HI testing. The Ab-bELISA demonstrated the presence of anti-BFDV antibodies (Figure 6.3). Except for bird 5, peak antibody titres of between 8.57% and 14.08% PI occurred between 2 and 4 weeks after the second inoculation (Figure 6.3). Bird 5 had a peak antibody titre of 20.42% at the second vaccination. These antibody levels, whilst low compared to levels found in experimentally infected birds, were all above the calculated negative cut-off value of 7% PI. Birds injected with the vaccine based on the truncated capsid sequence developed peak antibody levels at 2 weeks, 4 weeks earlier than birds which received the full-length vaccine. Repeated measures ANOVA found that the combined effect of vaccine type and time was significant (p<0.05). Paired t-tests between pre-vaccination samples and samples at each time point found that ELISA antibody levels were significantly different (p<0.05) at 4 and 6 weeks post-vaccination for the full-length and NLS-truncated vaccine, respectively.
6.4 Discussion
Presented here is the first report of the preliminary assessment of a DNA vaccine for BFDV. Due to the difficulty and expense associated with procuring BFDV-free psittacine birds, this study was only a small pilot trial, intended to assess whether the DNA vaccine constructs were expressed in vivo and resulted in seroconversion. Both the full-length and NLS-truncated DNA vaccines described above were successfully expressed and incited antibody development by vaccinated chickens. Although further work is required to optimise these DNA vaccines and information on the relative efficacy of each vaccine variant is limited, both DNA vaccines may be potentially useful candidates for the prevention of BFDV in psittacine birds. Because
of the debilitating course of PBFD, an effective vaccine against the virus is highly desirable and will significantly aid in the prevention and control of the disease. A killed whole-virus vaccine has been developed previously (Raidal and Cross, 1994a, Raidal et al., 1993a), but no cell culture method has been discovered to enable in vitro propagation of antigen or the development of infectivity assays. The antigen had to be purified from the feathers from persistently infected birds which produces a risk of incomplete inactivation of the virus (Raidal et al., 1993a, Wylie and Pass, 1987) as well as possibility of host protein contamination which could induce immune-mediated reactions.

Recombinant BFDV capsid proteins have recently been the focus of much research, as they can be produced in vitro in large quantities. Various research groups have developed and produced both full-length (Bonne, 2004, Heath et al., 2006, Stewart et al., 2007) and truncated (Heath et al., 2006, Johne et al., 2004) recombinant BFDV capsid protein and described their use as potential vaccine candidates and in serological assays.

DNA vaccines have the advantage over recombinant protein-based vaccines in that they are easier and cheaper to produce and plasmids encoding exogenous markers for the serological differentiation of vaccinated from naturally infected animals can be easily included. However, unless techniques of plasmid construction, vaccine preparation and injection are optimised, they may suffer from low levels of detectable antibody production after expression in vivo (Johnson et al., 2000) and may be less effective than recombinant protein vaccines (Blanchard et al., 2003). The presence of non-specific agglutinins in the sera of vaccinated chickens precluded measurement of antibody levels by HI and it is possible that some of the samples
with high bELISA antibody levels would have a detectable HI titre had these non-
specific agglutinins not been present. Several studies have shown that a positive HI
titre correlates strongly with freedom from disease (Khalesi et al., 2005, Raidal et al.,
1993b) and the bELISA used to evaluate the chickens’ antibody levels has been
shown to be both sensitive and specific and to correlate strongly with the HI test
(Section 5.3.4). Therefore, it is likely that if similar antibody levels were induced in
psittacine birds after vaccination with these DNA vaccines, that they would be
protected from infection.

This study evaluated the development and efficacy of two relatively simple DNA
vaccine constructs. There are a number of measures that can be used to improve
the efficacy of DNA vaccines in vivo, including gene electrotransfer (Dupuis et al.,
2000, Gao et al., 2007, Hojman et al., 2007, Mehier-Humbert and Guya, 2005),
codon optimisation (Garmory et al., 2003, Wang et al., 2006), inclusion of
immunostimulatory CpG motifs in the target sequence or backbone plasmid
(Garmory et al., 2003, Sato et al., 1996), encouraging muscle regeneration at the
injection site prior to vaccination (Davis and Whalen, 2004, Vitadello et al., 1994,
Whalen, 2004), administration of osmotic agents at the time of vaccination (Suarez
and Schultz-Cherry, 2000, Wu et al., 2004), and administration of adjuvants such as
interferons and interleukins (Li et al., 2004, Lillehoj et al., 2005). Any successful DNA
vaccination strategy will no doubt employ a combination of the above techniques.

The inclusion of extra immunostimulatory CpG motifs was not attempted in this
experiment because the capsid sequence of BFDV contains high proportions of CpG
motifs (Section 4.3.3.1). However, since CpG motifs can be both immunostimulatory
or immunosuppressive (Hasslung et al., 2003, Stevenson et al., 2006), identification
of the type of CpG motifs present in the BFDV capsid sequence and alteration of these (if necessary) to immunostimulatory sequences could improve the host response to the vaccine. If codon optimisation were to be attempted for a DNA vaccine for BFDV, though, one would need to optimise against the genome sequence of the chicken, as the genome sequence of any psittacine birds has not been described. Since the proportion of codons conserved between chickens and psittacine birds is unknown, evaluation of the relative efficacy of optimised and non-optimised DNA vaccines would require concurrent vaccination with each type of plasmid and subsequent measurement of antibody levels and response to live-virus challenge.

In this study, we attempted to improve the efficacy of the vaccines by preparing the muscle with bupivicaine (Davis and Whalen, 2004, Vitadello et al., 1994, Whalen, 2004). The peak antibody titre of bird 5 at the time of the second vaccination probably indicates that this was successful at this time in this one bird only and the peak is consistent with an initial IgM antibody response. Given the lower peak antibody titres of the other birds, muscle preparation was probably unsuccessful. Injection of the DNA vaccine in the exact place in the pectoral muscle that the bupivicaine was injected is very difficult; perhaps a more suitable site would be a smaller muscle such as the biceps brachii, triceps brachii, flexor carpi ulnaris or other suitable muscle of the wing.

Even though both vaccine variants resulted in the development of anti-BFDV antibodies, further evaluation and even modification of the constructs is desirable. Such work could include the assessment of the effect of NLS-truncation on the structure and function of the expressed protein (Birnbaum and Nassal, 1990, Heath
et al., 2006), inclusion of a secretory tag to enhance the availability of the expressed protein (Fan et al., 2008) and comparison between the DNA vaccines and recombinant proteins of the effects on cell-mediated immunity (Donnelly et al., 1997, Johnson et al., 2000, Toussaint et al., 2005). The effect of prior infection should also be assessed, as it has been shown that PCV2 infection can decrease the efficacy of vaccines (Opriessnig et al., 2006c).

In the field of vaccine research, the concept of heterologous vaccination, whereby different types of vaccines (modified live, killed, recombinant subunit and/or DNA vaccines) against multiple strains or serotypes are employed in order to incite concomitant humoral and cell-mediated responses, is becoming increasingly popular (Griebel et al., 2006, Toussaint et al., 2006, Toussaint et al., 2005, Wood et al., 2006). Co-administration of the full-length recombinant capsid protein may improve the expression of either of the DNA vaccine constructs described here, due to the ability of circovirus capsid proteins to bind DNA, enter antigen presenting cells and aid in its transport to the nucleus (Birnbaum and Nassal, 1990, Esko, 1999, Heath et al., 2006, Liu et al., 2001, Misinzo et al., 2006). Use of the full-length capsid protein in an heterologous vaccination strategy would also improve the immune response as a result of the immunostimulatory effect of virus-like particles (VLPs) (Noad and Roy, 2003, Tegerstedt et al., 2005, Ulrich et al., 1998). Whether or not the proteins produced in cells transfected with the DNA vaccines form VLPs and are processed as such would merit investigation, as would investigation of the difference in protection between vaccines utilising full-length and truncated proteins.
As BFDV is so genetically diverse, vaccines utilising the capsid sequence of multiple isolates would certainly be advantageous. DNA vaccination is an easier means of accomplishing this than producing multiple recombinant proteins. However given the number of species within the *Psittaciformes*, production of a truly heterologous vaccine would probably not be feasible. A combination of a few isolates, such as cockatoo, lorikeet, lovebird and budgerigar would likely be adequate and any heterologous BFDV vaccine should include the capsid sequence of the newly described cockatiel strain (Shearer et al., 2008a) which may be sufficiently different antigenically to qualify as a separate strain.

Other methods being investigated for their applications in both vaccination and gene therapy are replication-incompetent viral vectors (Ju et al., 2005, Minke et al., 2007, Pardoa et al., 2007, Poulet et al., 2003, Wang et al., 2007b), recombinant subunit vaccines produced in plants (Ma et al., 2005, Ma et al., 2003, Rigano and Walmsley, 2005) and artificially constructed viruses. These systems all have potential merit for use in vaccination strategies for both domestic and wild psittacine birds, but many aspects of function, protein expression, large scale production and efficacy still need to be addressed (Davis et al., 1993, Floss et al., 2007, Mastrobattista et al., 2005, Mastrobattista et al., 2006). Such systems could possibly be employed to vaccinate wild birds against BFDV, supplied in food and water at feeding stations, for example. However, testing and quality control would necessarily need to be extraordinarily strict and thorough to ensure, for example, that the replication-incompetent virus could not revert to virulence by recombining with a wild-type virus. In addition, there would need to be no negative effects on either target or non-target species, especially given the adverse effects identified in some human clinical trials (Check, 2003, Marshall, 1999).
Since there is no cell culture system available for BFDV, recombinant technologies likely hold the key to effective treatment and prevention options for the virus. In developing two novel DNA vaccines against BFDV, this study demonstrated that DNA vaccination is a potentially useful method of producing an effective vaccine. Effective vaccination strategies will likely include both recombinant proteins and DNA vaccines and should take advantage of new developments in immunology as the inclusion of avian-derived cytokines as both adjuvants and therapeutic agents.
Chapter 7. General Discussion

Despite the increasing volume of research on BFDV in recent years, many gaps remain in the knowledge of its mode of replication and its clinical and molecular pathogenesis. Efforts to develop diagnostic tests and vaccines for the detection of BFDV antigens and anti-BFDV antibodies that can be standardised and produced in bulk have been hampered by the lack of a cell culture system to grow the virus \textit{in vitro}. As such, investigations have largely focussed on the molecular (genetic and phylogenetic) characteristics of the virus and similarly detection has relied on PCR-based methods.

As previously discussed, detection of excreted virus and anti-BFDV antibodies requires the use of HA and HI assays, respectively. Because of the difficulties associated with implementing these tests (keeping a suitable flock of birds and preparing their blood) and ensuring consistency in their performance (due to differences in polyclonal antibody preparations and haemagglutination activity of donor erythrocytes), there are only a limited number of studies that have utilised them for investigation of the pathogenesis and epidemiology of BFDV (Khalesi et al., 2005, Raidal and Cross, 1994a, Raidal and Cross, 1995, Raidal et al., 1993a, Raidal et al., 1993b). Other laboratories have reported the development of these tests (Kondiah et al., 2005, Ritchie et al., 1991b, Sanada and Sanada, 2000, Soares et al., 1998) with similar or modified reagents, but have not reported their use in applied studies. Ongoing BFDV research requires consistent, high quality assays capable of detecting and quantifying viral nucleic acid, antigen and anti-BFDV antibodies in a range of samples. Molecular detection methods, whilst they only detect viral
nucleic acid and not viral antigen, are still extremely useful as they are based on reagents and equipment that are widely available and easily standardised.

As an antigen source, Cap is the most suitable as it has been shown to be the sole antigenic determinant (Stewart et al., 2007). Standard antibody preparations based on Cap will likewise have the most value for the development of diagnostic tests. However, it is suspected that antigenic differences exist between isolates and the research presented in Chapter 4 supports this theory. Any standard antibody preparations used in universal diagnostic tests should be tested against virus preparations from as many psittacine species as possible on an ongoing basis. There has only been 1 study to date investigating the possibility of the existence of BFDV serotypes (Khalesi et al., 2005) and while the authors could not find evidence of serotypes, antigenic mapping is necessary to definitively identify any antigenic variations between isolates.

For assays detecting viral nucleic acid, rep is theoretically the gene of choice as it is the most conserved part of the viral genome apart from the stem-loop, NLS and octanucleotide motif (Bassami et al., 2001, Khalesi et al., 2005, Raue et al., 2004). Assays for the detection of nucleic acid based on cap have been developed and seem to perform well (Albertyn et al., 2004, Ogawa et al., 2005, Raue et al., 2004). Theoretically the performance of each molecular assay will depend on the variation of the section of the genome that they amplify. Still, since none of these assays have been validated with a sufficiently large number of samples and their diagnostic performance has not
been compared with assays based on rep, they are less desirable choices for diagnostic assays.

The quantitative, real-time PCR assay described in Chapter 3 should be useful for the quantitation of viral DNA in blood samples from a range of psittacine species. It is based on a highly conserved section of rep, is able to detect virus from a range of psittacine species and has a greater analytical sensitivity than the standard PCR assay described by Ypelaar et al. (1999). Ensuring consistency in the quantitation standards, inclusion of reference genes into the assay for data normalisation and validation of the assay with other sample types, such as fresh and preserved tissues and swabs, should be pursued for the test to have as great an application as possible in both research and clinical diagnosis. A key finding of the experiment was evidence that vaccination does not prevent viral replication. The assay demonstrated that the viral load in both vaccinated and control birds increased for the first 4 weeks after challenge despite the fact that vaccinated birds had anti-BFDV antibodies before being challenged (Bonne et al., 2008b, Shearer et al., 2009).

Even though the assay measures viral DNA and not antigen, if studies are conducted to determine the infectivity of a given sample of virus (Coombs et al., 1993, Rusert et al., 2004) and correlated with the number of genome copies present, then the infectious load can be interpolated from the qPCR assay measurements. Even without this information, the assay could be used to fill in important gaps in the knowledge. For example, the kinetics of BFDV
infection and their correlation with the development and progression of antigen excretion, anti-BFDV antibodies and clinical signs in recently infected, chronically infected and apparently recovered birds.

Because Cap is the most suitable protein for the development of standard BFDV antigen and antibody preparations, a recombinant BFDV capsid protein and monoclonal antibody were produced as described in Chapter 5. Like other recombinant viral proteins (Noad and Roy, 2003, Tegerstedt et al., 2005, Ulrich et al., 1998), the full-length recombinant BFDV capsid protein self assembled into VLPs and importantly behaved like native BFDV in that it had HA activity and was recognised by naturally occurring anti-BFDV sera. Other recombinant BFDV capsid proteins have been developed (Heath et al., 2006, Johne et al., 2004) and have been shown to have merit for use in serological assays, but the morphology and haemagglutination behaviour of the proteins were not assessed.

Even though there have been no serotypes of BFDV described, there is still a possibility that monoclonal antibodies developed against the virus, or recombinant capsid proteins, may not detect all BFDV isolates because of variations in amino acid sequences between isolates. The monoclonal antibody developed in Chapter 5 detected viral antigen isolated from a cockatoo, a lorikeet and a cockatiel. Given that the cockatiel isolate has been shown to have some antigenic differences to other BFDV isolates, the fact that detection by the monoclonal antibody occurred indicates that the epitope it targets is likely to be conserved across virus isolates. Of course, ongoing
testing with as many virus isolates as possible and characterisation of epitopes present on the BFDV capsid protein is highly desirable. The other important property of the monoclonal antibody is the ability to inhibit haemagglutination. This means that the monoclonal antibody may be useful in the performance of HI assays. In addition, because the monoclonal antibody and recombinant protein can be quantified reasonably accurately, they could be used to measure and compensate for variations in erythrocyte haemagglutination sensitivity. The monoclonal antibody was also assessed to be useful in western blot and immunohistochemistry assays and should make these applications more user-friendly and more easily standardised.

Haemagglutination-inhibition assays are useful for the serological detection of exposure to BFDV and estimation of the amount of antibodies present in a sample. Antibody measurements are reasonably precise if attention is paid to minimising inter-assay variation by titrating virus and antibody activity against each other and against the erythrocytes from multiple birds prior to testing. Even so, HI assays are still prone to an appreciable amount of inter-test variation, especially if performed infrequently or without standard reference antigen and sera. Because of the lack of an easily standardised and widely applicable sero-diagnostic test, the Ab-bELISA developed in Chapter 5 should find wide application in both epidemiological surveys and routine sero-diagnosis. The Ab-bELISA is sensitive and specific, correlates well with the HI assay but has a greater analytical sensitivity. Like the HI assay, the Ab-bELISA is able to detect both IgM and IgY because the assay does not utilise a secondary anti-psittacine IgM/IgY antibody. Furthermore, since the
performance of the assay depends on the affinity of the sample antibody for the capsid protein, the type of animal from which the serum is collected does not matter and so the assay should be useful with sera from experimentally inoculated animals (e.g. mice, sheep or chickens).

The key advantage of the bELISA over an indirect ELISA is that it does not need a secondary antibody to psittacine IgY. Two studies have reported the use of anti-psittacine IgY in western blot (Heath et al., 2006, Johne et al., 2004) assays and one in an indirect ELISA assay (Johne et al., 2004). The antibody preparation described by Johne et al (2004) detected IgY from 7 diverse species of psittacine birds. Even so, the cross-reactivity of psittacine anti-IgY preparations has not been assessed and potential differences in affinity between samples could affect the result of tests using them. Additionally, the indirect ELISA reported by Johne et al (2004) was not validated with a sufficiently large number of samples, whereas the bELISA described in Chapter 5 has been validated with an acceptable number of samples (Jacobson, 1998). The bELISA was unfortunately not applicable to the detection of viral antigen, but the use of the same reagents in an antigen-capture ELISA, for example, should be easily accomplished and the resulting assay should be extremely useful.

As well as the need for new reagents and assays, there is the need to fill in the large gaps in knowledge concerning the genetic and antigenic spectrum of virus isolates, the interaction of the virus with a range of psittacine hosts and the pathophysiology of BFDV infections. Various phylogenetic analyses have
proposed the presence of “strains” of the virus, based on the species from which they were isolated, the characteristics of disease in those hosts and their geographical location (Bassami et al., 2001, Heath et al., 2004, Kondiah et al., 2006, Raue et al., 2004, Ritchie et al., 2003). While it is certainly likely that modifications to the virus will occur as a result of host-virus interactions, no genetic, antigenic or serological differences between these isolates have been identified or characterised.

The question of whether virus “strains” exist partly motivated the investigation described in Chapter 4. The discovery of BFDV infection in cockatiels is significant as infection had not been previously described in this species. The proposal that virus strains exist is partially supported by the fact that phylogenetic analysis grouped the cockatiel BFDV isolates separately to other isolates. Analysis of CpG content also suggests distinct differences in virus isolates, but the consistency of these differences within “strains” and their significance is yet to be investigated. Cross reactivity work demonstrated antigenic differences between the virus isolated from the cockatiels and other isolates, but the precise differences are unknown. Thus, questions of the existence of a novel BFDV serotype remain unanswered. Thorough investigation of the differences in pathogenicity of BFDV isolates and whether distinct strains exist will require epitope mapping, analysis of nucleotide sequence polymorphisms and recombination events and studies of infectivity using infectious clones on as wide a range of BFDV isolates and hosts as is possible, similar to experiments accomplished with the porcine circoviruses (Hughes and Piontkivska, 2007, Lefebvre et al., 2008, Ma et al., 2007, Mahe
et al., 2000, Meehan et al., 1998, Meerts et al., 2005a, Opriessnig et al., 2006d) and the VP2 protein of CAV (Wang et al., 2007a).

The existence of antigenic and nucleotide sequence differences between isolates is highly likely given the effects of random mutations (Nei, 2007), recombination (Biagini et al., 2001, He et al., 2007, Heath et al., 2004, Ma et al., 2007, Olvera et al., 2007, Worobey, 2000) and purifying selection (Hughes and Piontkivska, 2007). However, any suspected differences will need to be confirmed experimentally. Given the combined effects of these processes, the emergence of strains or even serotypes is possible (He et al., 2007, Heath et al., 2004, Hughes and Piontkivska, 2007, Nei, 2007, Olvera et al., 2007). This is especially likely considering the amount of substitutions that have accumulated in CAV (Nogueira et al., 2007) and PCV genomes (Olvera et al., 2007) and that PCV and CAV strains with different pathogenicity and cell tropisms have developed (Lucio et al., 1990, Meerts et al., 2005a, Nogueira et al., 2007, Opriessnig et al., 2006d). As such, the full spectrum of genetic, antigenic and pathogenic differences is unlikely to ever be fully described and characterised.

The reagents and tests developed as part of this research will have application in the investigation of the issues previously discussed and many other similar questions. For example, immunoblotting using the monoclonal antibody (and other monoclonal antibodies) may elucidate some antigenic differences between virus isolates, or could investigate the mechanisms of immunosuppression by detecting viral antigen in immunological cells and
tissues. The qPCR assay could be used to determine the viral load within various tissues and a modified qPCR assay could be used to measure cytokine mRNA expression in naïve and infected birds. The bELISA will be useful for sero-epidemiological studies. These types of studies should be more easily accomplished using the reagents and tests described in this study.

Some studies, however, will still require the use of preparations of infectious virus and live birds kept for long periods of time. Such key areas of knowledge include the cross-infectivity and pathogenicity of different virus isolates, the long-term progression of viral infection induced at various ages in different psittacine species, the individual variation in susceptibility to a given isolate and the effect of other diseases. These studies can be accomplished using the techniques already available, however their execution would benefit from the extra information available as a result of the new reagents and tests described in this study.

Investigations into the mode of replication, transcription patterns and infectivity of BFDV would be greatly enhanced by development of a system to culture the virus in vitro. To date, such efforts have been unrewarding, but the use of PHA and LPS to enable replication of TTV in cultured PBMCs (Desai et al., 2005, Mariscal et al., 2002) and IFN-γ to enhance PCV2 replication in PK-15 and monocytic cells (Meerts et al., 2005b) may indicate that such a system can be developed. Of course, recombinant technologies would still be the methods of choice for the production of vaccines and reagents for diagnostic
tests, but an in vitro culture system would make the isolation and characterisation of BFDV isolates much easier.

The usefulness of new highly sensitive and specific assays is limited unless the tests are applied properly and their results interpreted in a meaningful way. Any test, no matter how sensitive and specific, is vulnerable to false positive and negative results. Low disease prevalence and less than ideal methods for the prevention of cross contamination can result in false positives. BFDV testing is particularly susceptible to the occurrence of false positives as the virus is excreted in large quantities in feather dander which is easily aerosolised and manually transferred during handling. Our laboratory has seen instances of false positive PCR results occurring when clinicians have taken samples from a bird excreting BFDV in the feather dander and then taken samples from BFDV-negative birds. The small amount of virus transferred on the fingers is enough to give a positive reaction in the conventional PCR assay for at least 2 birds handled after the BFDV-positive bird (Sharp, 2007, pers comm).

Even widely used methods for sample collection and storage are susceptible to contamination. Bonne et al. (2008a) demonstrated that BFDV DNA can be transferred by hole-punch, with DNA detectable by conventional PCR in up to 5 pieces of filter paper processed after the original positive sample. False negative results are less affected by biological variables than false positives (Jacobson, 1998), so their occurrence is usually related to inhibitory effects or pipetting errors (Belak and Thoren, 2004). Nonetheless, if these errors occur,
they will affect the performance and validity of the assay and protocols should be in place to minimise the chance of their occurrence.

Worldwide, most laboratories rely on PCR methods for the diagnosis of BFDV infection because of the difficulty and expense associated with developing, maintaining and standardising HA and HI assays. This presents three problems, all with significant clinical implications. Firstly, as outlined above, improper sample collection and handling can give false results. Secondly, the detection of nucleic acid alone does not confirm active infection, merely the presence of the DNA of the organism. Furthermore, active infection does not necessarily result in clinical disease. Thirdly, a single test at a single point in time contributes little useful information on the complete clinical status and progression of the infection. Add to this the paucity of knowledge on the pathophysiology and progression of the disease and the results of a single test can be of limited value.

Even serological tests on their own tell us little about the patient’s infection status, merely whether the patient has been previously exposed to the infectious agent. Flock testing is a little better, but PCR testing alone will only show how many birds have virus present in the sample taken at that point in time. In comparison, serosurveys will at least indicate whether the flock has had recent exposure (low seroprevalence) or is endemically infected (high seroprevalence). With high prevalences worldwide and no easy and effective method of control, the best course of action is to conduct multiple tests (viral DNA, antigen and antibody) so that as complete a clinical picture as possible
is obtained. Tests such as the bELISA described in Chapter 5 and others based on similar standard reagents should make this much easier to accomplish. Ultimately, using a single test for an infectious agent is only warranted when the presence of the agent is cause for immediate action. This scenario occurs only when the prevalence of the agent is low as a result of effective, well planned and widely applied prevention strategies (vaccination and disinfection). For example, a parvovirus antigen test in a puppy with signs of gastrointestinal disease.

Effective control of BFDV will be greatly assisted by the availability of an effective vaccine. The lack of a system to grow the virus in cell culture means that recombinant technologies are the best way of producing such a vaccine. Chapter 6 details the methods used to construct two DNA vaccines based on cap. Both vaccines were expressed in vitro and elicited detectable antibody responses in chickens inoculated with the constructs, so they are attractive potential vaccine candidates. DNA vaccines are much cheaper and easier to produce than vaccines based on recombinant proteins or killed whole organisms and result in more potent cell-mediated immune responses (Johnson et al., 2000). However, unless they are administered using methods to improve their transfection efficiency and boost the immune response of the host, their effect may be suboptimal.
To date, only two studies have outlined efforts to characterise vaccines against BFDV and their protection of vaccinated birds from challenge with live virus. The first of these utilised a whole killed virus vaccine prepared from feathers and organs of PBFD-affected birds and whilst effective, the vaccine was too difficult and expensive to produce (Raidal et al., 1993a). Even though there have been a number of reports of the development of recombinant proteins as candidate vaccines (Bonne, 2004, Heath et al., 2006, Johne et al., 2004, Stewart et al., 2007), the difficulty associated with procuring and raising a flock of BFDV-free birds has meant that only one study thus far has evaluated the efficacy of a recombinant protein-based vaccine (Bonne et al., 2008b).

Both full-length and NLS-truncated proteins show promise in the development of effective vaccines, however recombinant protein vaccines utilising full-length capsid proteins are probably better candidates because the NLS will theoretically enhance the entry of the protein into antigen presenting cells (Esko, 1999) and its subsequent processing and presentation. Recombinant HBV proteins require only the first 144 amino acids for accurate self-assembly into VLPs (Birnbaum and Nassal, 1990), so NLS-truncated BFDV capsid proteins should behave similarly and should still confer protection from challenge with live virus, provided that antibodies to the NLS are not required for full protection. However, the contribution of the BFDV NLS to protection of vaccinated birds needs to be evaluated, as even though parvovirus B19 requires only the VP2 protein for formation of VLPs (Wong et al., 1994), the VP1 protein is required for the development of an effective immune response.
The in vitro expression of NLS-truncated BFDV capsid proteins has also been shown to be greater than that of full-length capsid proteins (Heath et al., 2006). The inclusion of a secretory tag in DNA vaccines utilising an NLS-truncated capsid gene of PCV2 has also been shown to significantly increase levels of protective antibodies (Fan et al., 2008). The effect these modifications have on the uptake, processing of and response to the various recombinant vaccines for BFDV remains to be investigated and it is likely that successful vaccination strategies will utilise a combination of approaches.

Further studies using vaccinated birds will be highly desirable to assess the length of protection conferred, whether birds are still protected after repeated challenge, how long maternal antibodies are protective against clinical disease in chicks and whether the progeny of vaccinated birds can still be vertically infected, as is the case with CAV (Brentano et al., 2005). The effect of pre-infection on vaccination should also be assessed, as PCV has been shown to decrease the efficacy of vaccines (Opriessnig et al., 2006c). The effect of circoviruses on vaccine efficacy is also likely to be economically significant in birds such as pigeons, geese and ducks, which are routinely vaccinated for other diseases. Should vaccines for other psittacine diseases be produced and implemented, the effects of BFDV infection on these vaccines will likely be significant. Evaluation of other vaccination strategies, such as viral vectors, plant expression systems and artificial viruses is also desirable.
Evaluation of the serological response to vaccines has previously required the use of the HI assay. Thus the bELISA described in Chapter 5, validated with samples from the vaccine trial (Bonne et al., 2008b), should prove to be an effective tool for the evaluation of future vaccines. The design of vaccines and related diagnostic tests should also ideally include strategies to differentiate infected from vaccinated animals (DIVA). Such strategies may include the development of a test to detect a viral protein not in the vaccine preparation (e.g. Rep for recombinant subunit or DNA vaccines), or a novel substance present only in the vaccine preparation (James et al., 2007). The production of recombinant infectious, but non-pathogenic, clones carrying genetic markers may also show promise for the development of vaccines with an in-built DIVA mechanism (Liu et al., 2007a). Thorough evaluation of the tests detecting viral proteins not present in vaccines is necessary in order to ensure that these tests are still valid if the bird is infected after vaccination. The prevalence of antibodies to the chosen viral protein or novel substance should be low or absent within non-vaccinated populations in order to prevent false positives and would also need to be evaluated thoroughly.

The availability of an effective vaccine will contribute immensely to the control of BFDV in both individual birds and avicultural collections. Administration of vaccines must be conducted in concert with appropriate quarantine and disinfection procedures for prevention and control programs to be effective. Based on studies with other non-enveloped viruses, peroxide compounds are theoretically the only products that can neutralise BFDV (Cross, 2006). Their effective use is hampered, though, by the difficulties associated with proper
cleaning of aviaries and the properties of the chemicals themselves. These products must be allowed to work on surfaces that are free of organic material and must be in contact with the surfaces for at least 10 minutes. Removing all virus-containing organic material and ensuring adequate contact time is very difficult and prolonged contact with peroxide compounds may damage stainless steel. The cleaning action associated with the use of many household bleach or ammonia compounds and specialised disinfectants (F10) will be useful for reducing the amount of virus present, but the compounds will not neutralise non-enveloped viruses. In Australia, exposure of captive birds to endemically infected wild psittacine flocks makes effective quarantine and disinfection especially difficult.

Effective quarantine to prevent the introduction or spread of BFDV is also very difficult given the lack of precise knowledge on the pathophysiology of the virus. It is strongly suspected that the virus is transmitted vertically, that recovered birds, especially lorikeets, eclectus parrots and king parrots, may revert to virus excretion despite the presence of circulating antibodies. In addition, BFDV isolates are probably infectious to all other psittacine species, but this is yet to be confirmed. Answering other questions of the pathophysiology of the virus will better inform the requirements for prevention and control. Such questions include the pre-patent period of the virus, whether birds only develop disease after being infected during the age-related window of susceptibility (Wylie and Pass, 1987) or whether birds infected as adults can also develop disease, whether birds that develop an appropriate antibody response can still develop clinical disease and whether particular
isolates cause different forms of clinical disease. Ongoing education campaigns are still required to reinforce the basics of quarantine to bird owners, breeders, traders and veterinarians, such as avoiding mixing large numbers of birds (e.g. at bird shows or pet shops) and preventing the premature introduction of new birds to the home or flock. Because of the ubiquitous and highly infectious nature of the virus, effective prevention and control strategies will require a combination of vaccination, thorough disinfection and strict quarantine, backed up by appropriate testing regimes and ongoing education.

Similar reasoning applies to the diagnosis, prevention and control of other diseases of companion birds. At present there are few routinely available assays for the diagnosis of common diseases. Where tests are available, they are mostly molecular-based methods and if serological tests are available, their interpretation can be confusing given that these diseases are often prevalent in the population. Most canine and feline infectious diseases benefit from years of research and prevention programs, so most veterinarians and owners are aware of the best ways to test for and prevent them. The gold standard tests for these diseases are chosen as such because they have usually been thoroughly tested and proven, whereas the owners and clinicians of avian patients often must choose a test because it is either the only one available or the best known. As research into BFDV and other avian diseases progresses, the manufacturers of such tests have a responsibility to ensure that the test or combination of tests provides the most useful clinical information possible, backed up by high quality research. Since the avian
market is relatively small, the danger exists that a well marketed test of poor quality would be better used than a less well marketed test of high quality, resulting in a disservice to all in the industry.

Treatment options are necessarily aimed at reducing viral replication or boosting the host’s immune response to the virus. RNA interference aims to do the former, whilst interferon therapy aims to do the latter. Only cytokine treatments have been trialled for BFDV infections and whilst promising, need to be evaluated more fully. Proper assessment of the success or failure of treatment trials will also depend on a more thorough knowledge of the pathophysiology of the virus as outlined above. Also important is the recognition that viruses are extremely adept at evading or modulating the immune system and as such any vaccination or treatment method is unlikely to completely eliminate the virus.

As has been demonstrated, vaccination does not prevent replication so logically virus may also persist after treatment. At least some of the immunosuppressive effects of the viral genome are probably independent of infectivity and interactions between viral and host proteins are unlikely to be disturbed if infectivity is lost (Kekarainen et al., 2008, Vincent et al., 2007). Therefore, clinical disease or secondary infections may still occur despite vaccination and/or treatment, especially after prolonged subclinical or repeated infections. Disruption of the IL-10 mediated immunosuppression may aid in recovery and viral clearance and is another option worth exploring. This could be accomplished through RNAi, specific anti-IL-10 monoclonal
antibody therapy or gene therapy (expressing a non-functional IL-10 mutant to compete with endogenous IL-10). As an aside, circoviruses also have potential as gene therapy vectors for the treatment of other diseases, since VLPs are easily produced and efficiently transported to the cell’s nucleus (Noad and Roy, 2003, Tegerstedt et al., 2005, Whittaker and Helenius, 1998).

There has been a need for widely available, efficacious and cost-effective vaccines and diagnostic tests for avian diseases for many years and the mainstream use and acceptance of recombinant technologies brings them a step closer. Equally important is the need to fill in key gaps in the information concerning the pathophysiology of BFDV and other avian diseases. The prototype diagnostic tests and vaccines described in this thesis will hopefully be of use in the development of cheap, commercially available tests and vaccines.

Turning the work described in this thesis into products available to the wider community will require much additional time, effort and money to scale up, validate and package them but this work should be rewarding and of great benefit. These products will be of use in research, pet bird health and the wider environment by assisting investigations into clinical and academic aspects of disease, maintenance of healthy pet birds and use in conservation programs, for example. The availability of these products will, in turn, work to promote further avian species’ status as companion animals. Just as vaccines for infectious diseases of dogs and cats help to control common diseases and involve owners in their care, vaccines for avian diseases will do the same for
birds. As these technologies and products are applied and accepted, owners’
and veterinarians’ involvement with birds will increase, as will their knowledge
of and care for them and they should be a valuable tool for the promotion of
avian health.
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### Appendix 1. Sequences used for phylogenetic analysis

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<tr>
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<td>AY450449</td>
<td></td>
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Beak and feather disease virus infection in cockatiels (Nymphicus hollandicus)

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Psittacine beak and feather disease is known to occur in a wide range of psittacine species; however, there are no scientific or credible anecdotal reports of psittacine beak and feather disease occurring in the cockatiel (Nymphicus hollandicus) despite it being one of the world’s most commonly kept companion bird species. Consequently, this has resulted in speculation that the species may have some innate resistance to beak and feather disease virus (BFDV) infection. To investigate this hypothesis we conducted a survey of cockatiels (n = 88) at commercial aviaries to investigate whether BFDV infection occurs in cockatiels, and found that all birds were virus-free by polymerase chain reaction and haemaggulutination assay and had no detectable antibody titre by haemaggulutination-inhibition assay. In addition to this, we sequenced the genome of two BFDV isolates obtained from diseased cockatiel feathers and performed cross-reactivity assays using virus eluted from these feathers and sera from naturally immune psittacine birds. Serological cross-reactivity results and phylogenetic analysis of the nucleotide sequences indicated that the cockatiel virus isolates were serologically and genetically different to other BFDV isolates. This is the first paper to report evidence of an antigenically distinct BFDV in psittacine birds.

Introduction

Beak and feather disease virus (BFDV) is the most common viral infection of psittacine birds, and the chronic debilitating feather disease that it causes has been confirmed in more than 60 psittacine species; it is highly probable that all are susceptible (Pass & Perry, 1985; Ritchie et al., 1989; Rahaus & Wolff, 2003; Albertyn et al., 2004). Surveys have been carried out in both wild and captive psittacine populations and reported virus prevalence rates vary between 10% and 94%, depending on the method of detection (McOrist et al., 1984; Raidal et al., 1993a; Rahaus & Wolff, 2003; Khalesi et al., 2005). Given the wide range of Psittaci-forme species reported to be susceptible, it is curious that the cockatiel (Nymphicus hollandicus) is greatly under-represented in both the scientific and lay literature. Indeed we know of no published reports of psittacine beak and feather disease (PBFD) in cockatiels even though the species is one of the most commonly kept companion bird species worldwide. It seems the only evidence of BFDV infection occurring in the cockatiel was a diagnosis made by polymerase chain reaction (PCR) in our own laboratory (Khalesi et al., 2005).

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

Materials and Methods

Samples. Feathers from two cockatiels (isolates 05-106 and 05-726) with characteristic skin lesions consistent with PBFD that had tested BFDV-positive by PCR and HA assay were used for subsequent PCR analysis and DNA sequencing as described below. A formalin-fixed feather follicle skin biopsy, submitted along with the feather of isolate 05-106, was also processed by routine histology methods, stained with haematoxylin and eosin, and examined by light microscopy for the presence of characteristic inclusion bodies (Figure 1a). BFDV infection was confirmed by immunohistochemistry (Figure 1b) as described below.

A survey of cockatiels in commercial aviaries was also conducted. Blood and feathers from 88 cockatiels at three commercial aviaries that had a laboratory-confirmed history of housing PBFD-affected psittacine birds were taken for testing. Blood was collected by venepuncture of the jugular or cutaneous ulnar vein and spotted onto filter paper (Whatmann, No. 3), then allowed to air dry at room temperature as described by Riddoch et al. (1996). Feathers were plucked and placed into clean 1.5-ml microcentrifuge tubes or zip-lock bags. PCR and HI assay was performed on blood, and feathers were used for HA testing.
Immunohistochemical staining using within macrophages in the feather pulp (arrow heads). Bar (Stewart primary monoclonal antibodies to recombinant BFDV capsid protein 3 min. Endogenous peroxides were quenched using 0.3% (v/v) H2O2 in slides, de-waxed three times in xylene for 3 min and re-hydrated using a digital camera accessory.

Immunohistochemical staining

Immunohistochemical staining using primary monoclonal antibodies to recombinant BFDV capsid protein (Stewart et al., 2007) and a horseradish-peroxidase-conjugated secondary antibody were performed on tissue sections from case 05-106. Briefly, 5 μm sections of formalin-fixed and paraffin-embedded feather tissue were cut using a Leica RM 2135 microtome, placed onto glass slides, de-waxed three times in xylene for 3 min and re-hydrated using decreasing ethanol concentrations and a final wash in Tris buffer for 3 min. Endogenous peroxides were quenched using 0.3% (v/v) H2O2 in methanol for 5 min and then washed in Tris buffer. Slides were incubated with a 1:50 dilution of the primary monoclonal antibody in Tris buffer for 10 min at room temperature, and unbound antibody was then removed by triplicate washes each for 3 min in Tris buffer before incubating with horseradish-peroxidase-conjugated EnVision anti-mouse (Dako) at room temperature for 30 min. The slide was washed as before and then antigen-antibody complexes were visualized with the chromagen diaminobenzidine (DAKO DAB Chromagen). Imaging was performed using an Olympus BX 13 microscope and a digital camera accessory.

Extraction of DNA from feathers

Viral DNA was extracted from feathers of the two cockatiels 05-106 and 05-726 using the methods described by Ypelaar et al. (1993b). Five millimetres of the feather calamus was cut on a sterile surface and placed into a microcentrifuge tube (Eppendorf). To this, 200 μl of 70% (v/v) ethanol was added and the tube vortexed briefly, and then the ethanol was removed and 200 μl sterile distilled water was added and the tube vortexed again. The sterile water was removed and 500 μl lysis buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.0), 2.5 mM MgCl2, 0.5% (v/v) Tween 20, 0.5% (v/v) Nonidet P40], containing 250 μg/ml proteinase K (Qiagen) was added. The feather in lysis buffer was incubated at 37°C for 1 to 2 h, before being heated to 95°C for 10 min. 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.

Polymerase chain reaction

The PCR was carried out using methods similar to those described by Ypelaar et al. (1999). Primers P2 (5’-AAC CCT ACA GAC GGC GAG-3’) and P4 (5’-GTC ACA GTC CTC TTT GTA CC-3’) amplify a 717 base pair (bp) fragment of open reading frame ORFV1 of BFDV DNA, and were used to test the surveyed cockatiels. Cockatiels 05-106 and 05-726 had previously tested positive for BFDV DNA using these two primers. Primers SeqP5 (5’-CTG CGA CCG TTA CCC ACA TA-3’) and SeqP10 (5’-TGG CCC TTT TCC GTT CCA AC-3’) were designed to amplify a 1479 bp fragment, the ends of which overlapped with the fragment generated by primers P2 and P4. Cockatiels 05-106 and 05-726 were tested using both primer sets; the two overlapping amplicons included the entire BFDV genome and were used in subsequent sequencing reactions. The PCR reaction consisted of 2 mM MgCl2, 5 μl of 5x polymerization buffer containing dNTPs, 12.8 pmol each primer and 0.1 U Tth-Plus DNA polymerase, made to a total volume of 25 μl using ultrapure water (all reagents Fischer Biotec). Reactions were carried out in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf). The PCR reaction using primers SeqP5 and SeqP10 was identical, except that 3 mM MgCl2 was used and the annealing temperature was lowered to 48°C. PCR products were visualized on a 1% agarose gel with the addition of 0.001% ethidium bromide, run at 90 V for 30 min.

Haemagglutination assay

The HA assays were carried out as described by Raidal et al. (1993b). Feathers were incubated with 100 μl phosphate-buffered saline (PBS) at 60°C for 1 h in a microcentrifuge tube (Eppendorf). The suspension was centrifuged briefly after incubation and 50 μl supernatant added to 50 μl PBS in a microtitre plate (Eppendorf). Serial dilutions of the solution were made to remove 50 μl solution from the first well and mixing with 50 μl PBS in the next well, and repeating the process across the row of the microtitre plate. Negative and positive control lanes were included for each batch of samples tested. The negative control consisted of PBS only, and the positive control was a 50 μl suspension of virus purified from feathers and organs of a BFDV-infected sulphur-crested cockatoo (Cacatua galerita), diluted to give a HA titre of 40 to 80 HA units/50 μl. To each well, 50 μl of a 0.75% to 0.85% (v/v) suspension of normal (non-haemagglutinating) galah erythrocytes was then added, and the microtitre plate was incubated for 1 h at 37°C. A positive HA titre was indicated by the suspension of the erythrocytes in the well of the microtitre plate, whereas a negative HA result was indicated by the sedimentation of the erythrocytes within the well.

Haemagglutination-inhibition assays

The HI assays were carried out as described by Raidal et al. (1993b) using antiserum purified from the feathers of a cockatoo with PBFD, diluted to give a HA titre of 4 to 8 HA units/50 μl. Plasma, serum or dried blood spots on filter paper were used for testing. Plasma or serum was first heat-inactivated at 57°C for 30 min, then 100 μl serum was added to 1 ml of 5% (v/v) acid-washed kaolin and the mixture incubated for 1 h at room temperature, or overnight at 4°C. Equal volumes of the supernatant was then haemadsorbed against a 10% (v/v) suspension of normal (non-haemagglutinating) galah erythrocytes for 1 h at 4°C. For blood collected onto filter paper, one 0.5-cm-diameter spot of blood on filter paper was cut out using scissors or a hole punch and collected into a microcentrifuge tube (Eppendorf). Then 100 μl of 5% (v/v) acid-washed kaolin was added and serum eluted from the paper by incubating the mixture for 1 h at room temperature, or overnight at 4°C. Then 50 μl supernatant was haemadsorbed against 50 μl of a 10% (v/v) suspension of normal (non-haemagglutinating) galah erythrocytes for 18 h at 4°C.

For testing, 50 μl haemadsorbed serum was added to 50 μl PBS in a microtitre plate well (Eppendorf). Serial dilutions of the solution were made to remove 50 μl solution from the first well and mixing with 50 μl PBS in the next well, and repeating the process across the row of the microtitre plate. Negative and positive control lanes were included.
for each batch of samples tested. The negative control was a 50 μl suspension of purified virus, diluted to give a HA titre of 4 to 8 HA units/50 μl. The positive control was a serial dilution of 50 μl chicken anti-BFDV polyclonal sera, processed as above. A positive HI titre was indicated by the sedimentation of the erythrocytes within the well of the microtitre plate, whereas a negative HI result was indicated by the suspension of the erythrocytes in the well.

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

Nucleotide sequence determination and analysis. PCR products were purified using an AxyPrep PCR cleanup kit (Axygen), according to the manufacturer's instructions. Dideoxynucleotide sequencing was carried out using an ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, except that the reaction volume was reduced to 10 μl and the annealing temperature used when sequencing the 1478 bp products was reduced to 50°C. Sequencing reactions consisted of 2 μl reaction buffer (containing Tris-HCl, MgCl2, fluorescently-labelled dNTPs and AmpliTaq DNA polymerase, concentrations not supplied; Perkin Elmer), 1 μl of 5x sequencing buffer (composition not supplied; Perkin Elmer), 10 to 20 ng of 717 bp PCR product or 20 to 40 ng of the 1479 bp PCR product, then made up to a total of 10 μl with ultrapure water (Fischer Biotech). DNA sequences were determined using an Applied Biosystems 3730 DNA Analyser and edited using Sequence Scanner v1.0 (Applied Biosystems) and GeneTool Lite (BTI Software). Edited sequences were analysed using MEGA 3.1 (Kumar et al., 2004). Neighbour-joining, maximum parsimony and Bayesian trees were constructed with 1000 bootstrap cycles for the neighbour-joining and maximum parsimony trees.

Results

Survey of cockatiels for evidence of BFDV and cross-reactivity assays. Of the 88 cockatiels tested using primers P2 and P4, none were positive for BFDV by PCR or HA assay and none had detectable antibodies to BFDV. None of the cockatiels surveyed had detectable HI activity against BFDV eluted from a cockatiel feather. Six of the eight known anti-BFDV HI-positive sera tested inhibited HA eluted from the feather of cockatiel 05-106, and similarly five of the eight sera inhibited HA eluted from isolate 05-726. Polyclonal chicken anti-BFDV antibody did not inhibit agglutination by either cockatiel virus isolate (Table 1).

Sequencing and analysis of PCR-positive samples from cockatiels. Primer sets P2/P4 and SeqP5/SeqP10 amplified overlapping 717 bp and 1497 bp fragments of the BFDV genome, respectively, from samples 05-106 and 05-726. Analysis of the sequences revealed that both sequences were 1993 nucleotides long and had identical features to other described circoviruses. A potential stem-loop structure, formed between bases 1976 and 1993 and between 1 and 12, as well as a repeated octanucleotide motif (GGGCACCG) were present immediately downstream of the stem-loop. Potential polyadenation signals were present in both sequences at identical positions: CATATAA between nucleotides 1019 and 1024 on the viral strand, and AATAAA on the complementary strand between nucleotides 758 and 763 (nucleotides 1231 to 1236 of the viral strand). A TATA box was also present, between nucleotides 86 and 89 of the viral strand. The area of the complementary strand between nucleotides 60 and 207, containing putative nuclear localization signals as described by Heath et al. (2004), was highly conserved across all sequences analysed, including the two cockatiel sequences.

Comparison of full-length sequences. Pairwise distances between cockatiel sequences and 26 other BFDV sequences varied between 3.2% and 15.5% (isolate 05-106) and between 3.8% and 14.5% (isolate 05-726) at the nucleotide level, and between 3.8% and 19% (isolate 05-106) and between 5.2% and 19.2% (isolate 05-726) at the amino acid level. Neighbour-joining phylogenetic analysis showed that both cockatiel sequences (isolates 05-106 and 05-726; GenBank accession numbers EF457974 and EF457975, respectively) clustered within cockatoo and galah isolates; however, maximum parsimony (data not shown) and Bayesian analysis grouped the cockatiel isolates distinctly separately, with a high posterior probability (Figure 2). Bayesian analysis also identified three distinct clades within the sequences (Figure 2); the cockatiels comprised clade number 1; the cockatoos and galahs plus three African grey parrots (AY521236, AY450443 and AY450435), a white-bellied caique (AY450434) and a Cape parrot (AY450439) made up clade number 2; and the remaining clade was made up of all Agapornis sp. isolates, the remaining African grey parrot and Poicephalus sp. plus a single rainbow lorikeet and a single Indian ringneck isolate.

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

<table>
<thead>
<tr>
<th>Antibody source</th>
<th>Cockatoo BFDV</th>
<th>Cockatiel 05-106</th>
<th>Cockatiel 05-726</th>
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<tr>
<td>Chicken anti-BFDV</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Red Lory</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
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<td>Long-billed Corella</td>
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<td>+</td>
<td>+</td>
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<td>Galah/Corella hybrid</td>
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<td>+</td>
<td>+</td>
</tr>
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<td>Rainbow lorikeet</td>
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<td>+</td>
</tr>
<tr>
<td>Short billed corella</td>
<td>+</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Short billed corella</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulphur crested cockatoo</td>
<td>+</td>
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A plot of CpG islands within the cockatiel sequences showed high proportions of CpG motifs throughout most of the sequence. Gardiner-Garden & Frommer (1987) defined a candidate CpG island as having $Y/C > 0.6$ on the CpG plot and a GC content $> 50\%$. As such, the first 30 bases and then bases 420 to 600, 640 to 1120, 1150 to 1570 and 1575 to 1993 of the complementary strand contained candidate CpG islands (Figure 3). Particular points of interest are spikes in the CpG plot at nucleotides 30 to 360, 630, 1120 and 1330 to 1540 of the complementary strand (Figure 3). The spikes at nucleotides 630, 1130 and the first spike between nucleotides 30 and 360 are less likely to be significant, however, as the GC content in these regions is less than 50\%. The whole sequence was GC-rich, as the GC content was only less than 50\% between bases 60 and 400, 580 and 640, 1120 and 1145 and 1550 and 1600 of the complementary strand (Figure 3). Analysis of the CpG and GC plots of the viral strand revealed similar findings but in different locations. Comparison of CpG plots derived from the cockatiel isolates demonstrated subtle differences compared with plots derived from published BFDV sequences from a sulphur-crested cockatoo (AF080560), a rainbow lorikeet (AF311299) and a peach-faced lovebird (AF311296). GC density plots for these isolates demonstrated minimal variation between the four isolates.

**ORFV1.** Sequences of both cockatiel isolates had a start codon of ATG located at position 131. The stop codon for both isolates was TGA, located at nucleotide 997. The predicted size of ORFV1 was 867 nucleotides. Distances between cockatiel sequences and other BFDV sequences varied between 2.5\% and 11.9\% (isolate 05-106) and between 2.4\% and 11.1\% (isolate 05-726) at the nucleotide level, and between 2.2\% and 10.4\% (isolate 05-726) at the amino acid level. Phylogenetic analysis showed that both cockatiel sequences clustered closest to, but separate from, the cockatoo and galah isolates (Figure 2).

**ORFC1.** Sequences of both cockatiel isolates had a putative start codon at nucleotide 16 (CTG) of the complementary strand (or nucleotide 1601 of the viral strand), as per Bassami et al. (2001). The stop codon for both isolates was a TAA at nucleotide 757 of the complementary strand (nucleotide 1235 of the viral strand). Distances between cockatiel sequences and other BFDV sequences varied between 2.0\% and 18.9\% (isolate 05-106) and between 5.9\% and 19.1\% (isolate 05-106) and between 6.0\% and 28.5\% (isolate 05-106) and between 6.8\% and 27.4\% (isolate 05-726) at the amino acid level. Phylogenetic
analysis showed that both cockatiel sequences clustered within a clade of cockatoo and galah isolates. Alignment of translated amino acid sequences showed that 121 of 260 amino acids were conserved across all the isolates examined, and 133 of 260 were variable. The areas between amino acids 68 to 83, 94 to 97 and 228 to 241 were especially variable, but the significance of this is unknown.

Discussion

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

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

The low reported incidence of BFDV infection in cockatiels and our negative serological survey results are somewhat puzzling for such a supposedly common and infectious virus that all Psittaciformes are presumed to be susceptible to. It is highly unlikely that the negative PCR results of all birds surveyed represent a failure of the test. Khalesi et al. (2005) reported only one false-negative PCR result from a total of 623 samples tested. The authors also showed that the PCR test is able to detect the cockatiel BFDV isolate, and this has been confirmed in the present study. It could be that all of the cockatiels surveyed in this present paper were naïve to infection, and were truly antibody negative. However, they were from commercial aviaries that had a high

Figure 3. CpG and GC plots of the complementary strand of the cockatiel (N. hollancicus) 05-106 isolate, overlain on CpG plots of BFDV isolates from a sulphur-crested cockatoo (C. galerita), a rainbow lorikeet (T. haematodus) and a peach-faced lovebird (Agapornis roseicollis) demonstrating areas of variable CpG regions at nucleotide 250 in the complementary strand.
turnover of a wide variety of psittacine bird species, many of which we knew from clinical observations were expressing clinical signs of PBFD. Typically the birds in such establishments have a high incidence of BFDV infection, PBFD and a high HI antibody prevalence, with budgerigars (Melopsittacus undulatus) and lovebirds (Agapornis spp.) having the highest rates of infection (Khalesi et al., 2005).

According to published epidemiologic data, an expected seroprevalence of 30% to 40% would be a conservative estimate (Raidal et al., 1993a; Raidal & Cross, 1994b; Khalesi et al., 2005) of the expected seroprevalence within the population of cockatiels that we sampled and a sample size of 88 should have provided a 95% level of confidence of the estimate of the prevalence of infection (Thrushfield, 1986). Failure to detect any evidence of antibody in such a sample size provides strong evidence (95% confidence level) that the seroprevalence in the population of cockatiels we sampled was less than 5% (Thrushfield, 1986). This is a very low figure in comparison with other Psittaciforme species. The lack of documented cases of cockatiels with PBFD in the literature along with the negative PCR and serological results obtained in our survey could be interpreted as evidence that cockatiels are somewhat innately resistant to BFDV infection. However, the PCR results in individual cockatiels reported here and by Khalesi et al. (2005) along with the histological evidence of BFDV infection (Figure 1) indicate that the species is susceptible to BFDV. Furthermore, the PCR results, DNA sequence analysis and HI cross-reactivity data provide evidence of a cockatiel-adapted BFDV that may be sufficiently different, genetically and antigenically, from most other BFDV isolates to be considered a separate strain of the virus. This is not surprising given evidence that avian circoviruses have coevolved with their host species (Ritchie et al., 2003; Stewart et al., 2006). Psittaciforme mitochondrial DNA phylogeny has placed the monotypic Nymphicus more closely related to the black cockatoos (Calyptrorynchus and Callocephalon), and not the more distantly related white cockatoos (Cacatua and Eolophus) as was once thought (Brown & Toft, 1999), and the results presented in this present paper provide further support to this hypothesis.

Given the data presented in this paper, there are some aspects of BFDV evolution and epidemiology that are difficult to explain. Based on the phylogenetic analysis (Figure 2), the cockatiel BFDV sequences are genetically distinct from those obtained from other psittacine species. However, the same method of analysis and other similar studies have also found similar distinct genetic differences between BFDV isolates from lovebirds, lorikeets and cockatoos. In addition, cross-reactivity work using BFDV eluted from the feathers of two rainbow lorikeets, a musk lorikeet (Glossopsitta concinna), a red lory, two swift parrots (Lathamus discolor), a sulphur-crested cockatoo and a scarlet chested parrot (Neophema splendida) and anti-BFDV antibodies from the same panel of birds as in this study has shown these not to be serologically distinct (Khalesi et al., 2005), and therefore it is difficult to clearly associate a specific mutation or genetic variation in the cockatiel sequences with any biological or antigenic characteristics.

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

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

References


A quantitative, real-time polymerase chain reaction assay for beak and feather disease virus

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Abstract

PCR-based assays for the detection of BFDV DNA are in widespread use throughout the world. 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 can quantify the amount of DNA present in a sample. This study describes a quantitative real-time PCR assay for the detection of BFDV DNA, using primers designed to amplify a conserved 81 bp fragment of ORFV1 and SYTO9, a fluorescent intercalating dye. A synthetic oligonucleotide was used to make standard curves for the quantitation of viral load in blood and feather preparations. The assay was very sensitive, with a detection limit of 50 copies/µL. The assay was developed using DNA extracts from the feathers of 10 different species of birds which had tested BFDV-positive previously and was validated with blood and feather samples from corellas vaccinated with an experimental BFDV vaccine, then challenged with live virus. Viral DNA was detected consistently in the blood of all control (non-vaccinated) birds and in some vaccinated birds. Contamination of the environment with feather dander from BFDV-infected birds meant that feather preparations used for the haemagglutination assay were unreliable for the detection and quantitation of viral excretion. Nonetheless, the assay should prove to be a useful and sensitive test for the detection of viral DNA in a range of samples.

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1. Introduction

Psittacine beak and feather disease (PBFD) is the most common viral disease of psittacine birds and causes either a chronic debilitating feather disease in adult birds (Albertyn et al., 2004; Pass and Perry, 1985; Rahaus and Wolff, 2003; Ritchie et al., 1989b) or a severe, acute disease syndrome in nestlings and African Grey parrots (Psittacus e. erithacus) (Doneley, 2003; Raidal and Cross, 1995; Schoemaker et al., 2000). The causative agent, beak and feather disease virus (BFDV), is a circovirus with a single stranded DNA genome, approximately 1.7–2.0 kb in length (Bassami et al., 2001; Ritchie et al., 1989b) and is considered to have a worldwide distribution (Albertyn et al., 2004; Hsu et al., 2006; Kiatipattanasakul-Banlunara et al., 2002; Kock et al., 1993; McOrist et al., 1984; Pass and Perry, 1985; Rahaus and Wolff, 2003; Ritchie et al., 1989a). 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 in this study is a novel qPCR assay for the detection and characterisation of BFDV infection.
2. Materials and methods

Insofar as was practical, the qPCR was developed and validated in accordance with guidelines established by the Office International des Epizooties (O.I.E.) for the development of PCR methods for the diagnosis of infectious diseases (Belak and Thoren, 2004; Jacobson, 2004).

2.1. Samples

DNA extracts used for the development of the qPCR were taken from the archive of samples tested previously 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 (Poephila gulielmi), green-cheeked conure (Pyrhura molinae), sun conure (Aratinga solletittalis), yellow-tailed black cockatoo (Caloptyronus funereus) and cockatiel. After DNA extraction as described below, samples were stored at −20 °C for up to 5 years.

To validate the qPCR, samples from eastern long-billed corellas (Cacatua tenuirostris) that were part of an experiment to assess a recombinant BFDV capsid protein-based vaccine (Bonne et al., 2008b; Stewart et al., 2007) were tested using the optimised qPCR assay. Long-billed corellas received 1 mL vaccine containing 10 mg recBFDVcap by intramuscular injection on day 0 and 0.4 mL vaccine containing 66.8 mg recBFDVcap on day 11. All vaccinated corellas and five non-vaccinated control corellas were given 0.4 mL BFDV suspension [titre = log10 12 haemagglutination units (HAU)] per 50 µL intramuscularly and 0.1 mL orally 16 days after booster vaccination. Samples of blood and serum or plasma, as described above, were taken from vaccinated (n = 15) and non-vaccinated control (n = 5) birds on six separate occasions, including the time of vaccination and the time of challenge with live virus. Not all samples were available at each collection, so in total 108 samples were thus collected. As part of the vaccination trial (Bonne et al., 2008b), blood samples were tested by HA (Raidal et al., 1993) and PCR (Ypelaar et al., 1999) assay. Crude DNA extracts were also made from the corresponding feather eluates prepared for HA testing (Bonne et al., 2008b; Raidal et al., 1993) and 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 5). As part of the vaccination trial (Bonne et al., 2008b; Raidal et al., 1993) and PCR (Ypelaar et al., 1999) assay. Crude DNA extracts were also made from the corresponding feather eluates prepared for HA testing (Bonne et al., 2008b; Raidal et al., 1993) and tested using the optimised qPCR assay.

Viral DNA was extracted from feathers using the methods described by Ypelaar et al. (1995). Five mm of feather calamus was cut on a sterile surface and placed into a microcentrifuge tube (Eppendorf, Hamburg, Germany). 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 was 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, Hamburg, Germany) was added. The feather in lysis buffer was incubated at 37 °C for 1–2 h, then being heated to 95 °C for 10 min. The solution was centrifuged and DNA was extracted from the supernatant with the Qiagen blood mini kit (Qiagen, Hamburg, Germany), 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 60 °C for 1 h in a microcentrifuge tube (Eppendorf, Hamburg, Germany). The solution was centrifuged briefly after incubation and 10 µL of the supernatant transferred to another microcentrifuge tube, then boiled for 10 min.

2.3. Extraction of DNA from dried blood spots

Viral DNA was extracted from dried blood spots using Qiagen blood mini kit (Qiagen, Hamburg, Germany), 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, Hamburg, Germany). 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 85 °C for 10 min, then centrifuged. Twenty microlitres of proteinase K (Qiagen, Hamburg, Germany) were added to the tube, then the tube was vortexed and incubated at 56 °C for 1 h. After incubation, the solution was centrifuged and DNA extracted from the supernatant according to the manufacturer’s protocol.

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′-GAG CCG ATT AAG GAA G-3′) and P6 (5′-TAG CGA GGT ATG CAA GC-3′) (Geneworks, Hindmarsh, Australia) were designed to amplify an 81 bp 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 MgCl2, 5 µL of 5× polymerisation buffer containing dNTPs, 3.34 µM SYTO9 fluorescent dye (Invitrogen, Carlsbad, CA), 12.8 pmol of each primer and 0.1 U of Tth Plus DNA polymerase, ultrapure water in a total volume of 23 µL (all reagents Fischer Biotec, Perth, Australia, 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 CCC GGA CCC AAA ATG AAG GAA GAA GTC GCC CCT GCT TTA CAA ATG TCC AGG CCG GGT CCG GCC GTT CAT AAT TCC TAC TGC AGG CCG GGT CCG GCC GTT CAT AAC CTC TCG CTA TGG TGT-3′) (Geneworks, Hindmarsh, Australia). The oligonucleotide was diluted to an initial concentration of 1 × 1012 copies/µL, then serially diluted 1:100 to give standards of 1 × 1010, 1 × 109 and 1 × 108. The 1 × 106 standard was serially diluted 1:10 to give standards of 1 × 105, 1 × 104, 1 × 103, 1 × 102 and the 1 × 102 standard diluted 1:2 to give a standard of 50 copies/µL. Two 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, Concorde, Australia) real-time thermocycler. Cycling conditions consisted of an initial denaturation at 95 °C for 5 min, then 40 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 20 s, followed by a final extension step at 72 °C for 10 min.

2.5. Melt curve analysis

A melt curve analysis was performed after each run, with the mixture being cooled to 60 °C for 45 s then heated in one-degree increments to 95 °C. Fluorescence was measured for 15 s at each increment and a graph plotting the second derivative of the melt
of variance 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 10 copies/μL, as the coefficient of variation of the 10^2 and 10^3 standards was close to 100% 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 (Fig. 1). The geometric mean viral load ± S.E. in feather extracts of control birds was estimated at 10^{3.549052655} ± 10^{0.4240215976} copies/μL at 2 weeks post-challenge, rose to a peak of 10^{6.76052006} ± 10^{3.42709188} copies/μL (range 10^{4.31455309} to 10^{5.53724649} copies/μL) at 4 weeks post-challenge, then decreased to 10^{4.585202596} ± 10^{2.05913079} copies/μL (range 10^{3.633698727} to 10^{6.29587779} 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 detected in the feather extracts of 3 vaccinated birds at 2 weeks post-challenge, 6 vaccinated birds at 4 weeks post-challenge and 10 vaccinated birds at 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 10^{1.9709454} and 10^{6.6237159} copies/μL). The samples which were positive on qPCR of feather extracts but negative on blood samples were considered to be false positives. The results of point-biserial analysis of the correlation between the qPCR assay and the standard PCR assay at various time points were not statistically significant (p > 0.05). Analysis of the correlation between the qPCR assay and the HA assay was not performed, since virus was detectable by HA in the feather samples of only one control bird at 4 and 6 weeks post-challenge. 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) (Fig. 2).

Two sets of comparisons were made for the calculation of sensitivity and specificity. Firstly, similar tests were compared. For blood samples, the standard PCR assay was considered to be the gold standard assay and was used for comparison with the results of the qPCR assay using blood samples. For feather eluates, the HA assay was considered to be the gold standard assay and was compared with the results of the qPCR assay which used DNA extracted from HA samples. Sensitivity was 94.73% and specificity 87.61%, with positive and negative predictive values of 56.25% and 90%, 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 sam-
Fig. 2. 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 and (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. Discussion

Given the high prevalence of BFDV infection worldwide, sensitive and specific detection methods which use standard methods and consistent reagents are both highly desirable and necessary. The qPCR assay described in this study has a very low limit of detection 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.

Although it is well accepted that qPCR assays have a much greater analytical sensitivity than standard PCR (Mackay, 2004; Wong and Medrano, 2005), currently the standard PCR assay is the gold standard for the detection of BFDV nucleic acid and hence the comparative diagnostic sensitivity and specificity were determined. 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 negative by standard PCR assay. However, because of this fact the analytical sensitivity could not be determined precisely. Importantly, in this study it was impossible to differentiate between true positive results below the limit of detection of the HA and standard PCR assays and false positive results. In this study the effect is likely to be minimal since there was accurate information on the infection status of the birds. The comparative diagnostic sensitivity and specificity was good, however because of the greater analytical sensitivity of the qPCR, the absolute diagnostic sensitivity and specificity is likely to be higher. 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 were within values accepted by the OIE (Jacobson, 1998); reproducibility could not be assessed since testing in other laboratories was impractical. Negative predictive values were uniformly high 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. The effect of false positive results cannot be ignored but remains to be elucidated. When comparing the HA assay with the 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. The assay should still be useful for testing samples from naturally infected birds, provided that appropriate samples are collected and properly stored and processed.

Not only did the qPCR assay detect viral DNA in birds that tested negative by the standard PCR (Bonne et al., 2008b), but also it demonstrated that the viral load in both vaccinated and control birds increased for the first 4 weeks after challenge. Vaccinated birds had anti-BFDV antibodies before being challenged (Bonne et al., 2008b; Shearer et al., 2008), thus vaccination did not prevent viral replication. This is a common scenario with other vaccines (Opriessnig et al., 2006) and is not surprising in this case. Given this fact, it is likely that the chicks of vaccinated birds may still be vertically infected despite the presence of antibodies in the parents, as occurs with PCV2 and CAV (Brentano et al., 2005; Larochelle et al., 2000). 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. Prevention of vertical infection would be an important measure in reducing the incidence of PBFD and consequent
secondary infections over a bird’s lifespan. Of course, maternal antibody levels will wane soon after hatching and so horizontal infection is still probably the primary route of infection.

An absolute method of quantitation was selected for this assay, as it was useful to assign a set quantity to measurements. No quantitation method is foolproof, however and both absolute and relative quantitation methods are both legitimate, provided that the assay itself is properly validated (Peirson et al., 2003; Sellar et al., 2007). 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 genomics controls (Hendriks-Balk et al., 2007). The data in this study were not normalised to a reference gene, or housekeeping, gene as candidate genes for normalisation of gene expression data in psittacine birds have not been evaluated. In fact, there have been surprisingly few studies investigating the suitability of avian reference genes (Li et al., 2005; Yamashita et al., 2007). Normalisation is also more important when testing for cell-associated virus and this study was concerned with the detection of circulating and excreted virus. 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. Appropriate control genes for qPCR assays used to study BFDV will require studies to account for disease states, the tissue(s) sampled and the species of host psittacine (Li et al., 2005; Maccoux et al., 2007; Radonić et al., 2005; Robinson et al., 2007; Spinanski et al., 2006; Sturzenbaum and Kille, 2001; Tang et al., 2007; Tricarico et al., 2002). 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. In practice, this may add extra cost and complexity to the assay and any such benefits gained from data normalisation would need to be determined.

Because of the high degree of genetic variation between BFDV isolates (Bassani et al., 2001; de Kloet and de Kloet, 2004; Heath et al., 2004; Konidaria et al., 2006; Ritchie et al., 2003) intercalating dye, in which the presence of the pathogen(s) is indicated by the melting peaks of the PCR product. The qPCR could also be used to determine the amount of infectious virus in a sample once infectivity bioassays are conducted to determine the ID50 of BFDV as has been done for HIV-1 (Coombs et al., 1993; Rusert et al., 2004).

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. Variations in erythrocyte, polyclonal antibody and virus 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. 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 of HA and HI assays (Johne et al., 2004). However, in regards to PCR assays, 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 also introduce unacceptable levels of variation and render a test diagnostically and clinically useless (East et al., 2004; Bonne et al., 2008a; Knuottson et al., 2004). 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 in a single sample does not necessarily indicate active infection. Non-replicating DNA may be present in blood for up to 3 months (Lazizi and Pillot, 1993) and seropositive birds may still be PCR positive whilst they are clearly infected (Khaledi et al., 2005). Testing of these birds by PCR alone would miss this important piece of information. Quantitative assays for detection of nucleic acids are less likely to lead to false clinical assumptions since they report the amount of nucleic acid present rather than just its existence. Care should still be taken in interpreting results when contamination is likely or when the amount of detected nucleic acid is low, though. Nonetheless, provided these factors are considered, the qPCR assay described in this study should prove to be a sensitive, specific and accurate diagnostic tool for the detection and characterisation of BFDV infection.

Acknowledgements

We wish to thank Dr. Andrew Mikosza (Murdoch University) for his advice regarding qPCR assays. Funding for this research was provided by grants from the Natural Heritage Trust and Murdoch University.


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

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Article history:
Received 17 November 2008
Received in revised form 28 January 2009
Accepted 5 February 2009
Available online 14 February 2009

Keywords:
Beak and feather disease virus
Blocking ELISA
PBFD
Circovirus
Serology

ABSTRACT
Currently, the only diagnostic test available routinely for the sero-diagnosis of BFDV is the haemagglutination-inhibition (HI) assay. This test, whilst useful and applicable to samples from a wide range of psittacine birds, is not an ideal assay; it requires erythrocytes from live animals, virus purified from the feathers of infected birds and polyclonal antibody preparations in order to perform the assay. Variations in these reagents make consistency between tests difficult to achieve, underscoring the need for a new test with standardised reagents for the sero-diagnosis of BFDV infection which has led to the development of an antibody response. The methods used to develop a novel “blocking” (or “competitive”) ELISA (bELISA) for the detection of anti-BFDV antibodies in psittacine sera are presented in this paper. The assay was developed using a baculovirus-expressed recombinant BFDV capsid protein and a newly developed monoclonal antibody raised against this protein. The assay was then validated with 160 samples from eastern long-billed corellas (Cacatua tenuiostris) vaccinated with the recombinant capsid protein and challenged with live virus and samples from 82 cockatiels known to be HI negative. The bELISA described in this study is a sensitive and specific diagnostic test and should have wide application for the sero-diagnosis of BFDV.

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1. Introduction
Psittacine beak and feather disease (PBFD) is the most common viral disease of psittacine birds and causes either a chronic debilitating feather disease in adult birds (Albertyn et al., 2004; Pass and Perry, 1985; Rahaus and Wolff, 2003; Ritchie et al., 1989b) or a severe, acute disease syndrome in nestlings and African Grey parrots (Psittacus e. erithacus) (Doneley, 2003; Raidal and Cross, 1995; Schoemaker et al., 2000). The causative agent, beak and feather disease virus (BFDV), is a circovirus with a single stranded DNA genome, approximately 1.7–2.0 kb in length (Bassami et al., 2001; Ritchie et al., 1989b) and is considered to have a worldwide distribution (Albertyn et al., 2004; Hsu et al., 2006; Kiatipattanasakul-Banlunara et al., 2002; Kock et al., 1993; McOrist et al., 1984; Pass and Perry, 1985; Rahaus and Wolff, 2003; Ritchie et al., 1989a). Surveys using PCR-based assay methods have found prevalence rates of between 8% (Bert et al., 2005) and 39% (Rahaus and Wolff, 2003). The reported seroprevalence varies between 16% and 62% (Khalesi et al., 2005; Raidal and Cross, 1994) among captive flocks and between 41% and 94% in wild flocks (Raidal et al., 1993a).

Currently, a haemagglutination-inhibition (HI) assay (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 is useful detects both IgM and IgG 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, a novel blocking (or competitive) ELISA has been developed which utilises a baculovirus-expressed recombinant BFDV capsid protein (Stewart et al., 2007) and a newly developed monoclonal antibody raised against this protein (Shearer et al., 2008b).

2. Materials and methods

Insofar as was practical, the bELISA was developed and validated in accordance with guidelines established by the Office International des Epizooties (O.I.E.) for the development of serological assays for the diagnosis of infectious diseases (Jacobson, 2004).

2.1. Samples

Samples of blood, collected on filter paper and air-dried and serum or plasma from 5 species of psittacine birds (including

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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, Frickenhausen, Germany) and allowed to coat at 4 °C overnight. The plate was then washed with wash buffer, blocking buffer added to all wells and the plate incubated for 1 h at room temperature. After another wash step, 50 μL of blocking buffer was added to all wells of the ELISA plate. Then 50 μL sera from each of the 5 psittacine birds described above, eluted as previously described, was added to the first well of each row and serially diluted across the plate. The plate was then incubated for 1 h 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, diluted 1:400 in blocking buffer, was added and the plate incubated for 1 h at room temperature. After washing, 50 μL of HRP-conjugated polyclonal anti-mouse IgG (Sigma–Aldrich, St. Louis, USA), at the manufacturer’s recommended dilution in blocking buffer, was added to each well and the plate incubated for 1 h at room temperature. After washing, 50 μL/well of a solution containing ABTS (BioRad Laboratories, Hercules, USA) was added and colour allowed to develop for 15 min at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405 nm 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.

Validation of the bELISA

To validate the bELISA, 160 samples from eastern long-billed corellas (C. tenuirostris) vaccinated with a recombinant BFDV capsid protein (Bonnie et al., 2009; Stewart et al., 2007) and 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, samples from 82 cockatiels that had been assessed as negative by HI (Shearer et al., 2008a) were also tested using the optimised bELISA.

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, Hamburg, Germany) and allowed to coat at 4 °C overnight. The plate was then washed with wash buffer (PBS, 0.05% (v/v) Tween 20, 5% (w/v) skim milk powder (Fonterra Foodservices, North Ryde, Australia)) 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 h at room temperature. Serum or plasma samples were diluted 1:5 in blocking buffer.

2.3. Optimisation of antigen and antibody dilutions using indirect enzyme-linked immunosorbent assay

The indirect ELISA was optimised as described by Shearer et al. (2008b). 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 0.05 M carbonate/bicarbonate buffer, applied to duplicate 12-well rows of two Microlon 600 ELISA plates (Greiner BioOne, Frickenhausen, Germany) and allowed to coat at 4 °C overnight. The plate was then washed with wash buffer (PBS, 0.05% (v/v) Tween 20), blocking buffer was added to all wells and the plate incubated for 1 h 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 were added to all wells of each plate. Then 50 μL of a 1:25 dilution of the monoclonal antibody were added to the first well of the first row of each protein dilution and 50 μL of 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, leaving 50 μL of each dilution per well and the plates incubated for 1 h at room temperature. After washing again, HRP-conjugated goat anti-mouse IgG (Sigma–Aldrich, St. Louis, USA), at the manufacturer’s recommended dilution in blocking buffer, was added to all wells of both plates and the plates incubated for 1 h at room temperature. The plates were washed again, then 50 μL/well of a solution containing ABTS (BioRad Laboratories, Hercules, USA) was added and colour allowed to develop for 15 min at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405 nm measured using a spectrophotometer.
oclonal 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.

3.2. A novel blocking ELISA for the detection of antibodies to BFDV

Sera from each of the 5 species of psittacine birds with naturally occurring anti-BFDV antibodies tested positive by bELISA, as did the sera from the chicken inoculated with the recombinant BFDV capsid protein. The optimal dilution of these sera 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 (Fig. 1). Non-vaccinated control birds had peak antibody levels of between 55.25% and 83.26% after challenge with live virus. Importantly, although the samples from birds vaccinated with the recombinant protein were HI-negative at the time of the second vaccination, the bELISA detected low levels of antibodies in these samples. The correlation coefficient between antibody levels measured by bELISA and HI titres was high ($r^2 = 0.8156$, $p < 0.05$) (Fig. 2). 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 (Fig. 3a). 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 (Fig. 3b).

4. Discussion

At present, HI and HA assays are the best available assays for the detection of anti-BFDV antibodies and excreted virus, respectively. However, given the amount of variation inherent in both the HI and HA assays, novel diagnostic tests for both the virus and the presence of anti-BFDV antibodies in serum are highly desirable. Presented in this paper 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 and have the distinct advantage over indirect ELISAs 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 sensitivity and specificity of the blocking ELISA developed in this study were within values defined as acceptable by the OIE (Jacobson, 2004) and should prove to be a useful diagnostic test for BFDV. The analytical sensitivity was greater than the HI test, as it detected antibodies in vaccinated birds that were HI negative and 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, 160 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 1014. 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 were within values accepted by the OIE (Jacobson, 1998). The cut-off value was determined 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, the 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 correlation coefficient between bELISA values and HI titres from the vaccinated corellas (Bonne et al., 2009) was high, which is somewhat surprising given the differing nature of the two tests. The HI assay gives a defined cut-off, whereas the bELISA allows for continuous readings of PI values for samples with antibody levels which fall in between HI end-points. This difference means that although the correlation coefficient was high no direct inference between bELISA PI values and HI titres can be made as each HI end-point titre can be associated with a range of bELISA PI values (Fig. 2). Thus, the bELISA is the preferred antibody test, as it allows more precise characterisation of antibody levels. 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 (John 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 392 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 in this article, 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 in this article has been tested with sera from 6 different species of psittacine birds and validated with 160 samples, further testing is necessary to ensure that the assay performs well with sera from as many species of psittacine birds 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., 2008a).

The monoclonal antibody used in this study recognised BFDV from a cockatiel, sulphur-crested cockatoo and a rainbow lorikeet (Shearer et al., 2008b). This indicates that there is some antigenic homology between isolates, even if the cockatiel BFDV isolate is indeed a separate serotype. Whilst it is possible that the epitope detected by this monoclonal antibody is not present in some BFDV isolates, the fact that no serotypes have been discovered to date suggests that enough major epitopes should be conserved between isolates that polyclonal anti-BFDV sera will react with the recombinant protein. Bound sera will thus block the monoclonal antibody from binding, even if the test serum does not react with the exact epitope detected by the monoclonal antibody. Thus, the bELISA should be reliable for use with sera from many species of psittacine birds, unless experimental evidence comes forth that novel BFDV serotypes exist or that the epitope detected by the monoclonal antibody is not immunodominant. 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.

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 more 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, polyvalent 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 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, HI assays are still prone to an appreciable amount of inter-test variation.
Other advantages of the bELISA over the HI assay are reduced sample preparation time and the potential to shorten the time taken to perform the assay if plates are pre-coated and blocked in bulk, or if the monoclonal antibody is directly conjugated to HRP. However, the effect of these modifications on assay performance 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 in this paper 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.

Acknowledgement

Funding for this research was provided by grants from the Natural Heritage Trust and Murdoch University.

References