Final Report for the Australian Government Department of the Environment and Heritage

Development of Recombinant Proteins as a Candidate Vaccine for Psittacine Beak and Feather Disease

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Non-technical Summary

The main aims of the project that Murdoch University undertook included:

1. Develop a Beak and Feather virus using bacteria.
2. See if antibodies recognise the engineered virus from naturally infected bird species.
3. Determine if the engineered virus will induce an antibody response in sheep?
4. And will these antibodies recognise native virus?
5. Will psittacine species antibodies respond to the engineered virus?
6. Identify and prioritise the gaps in existing knowledge relating to this field of research and provide recommendations as to areas of future research activity that will address those gaps identified.

This research project successfully engineered a Beak and Feather Disease virus that was recognised by anti-BFDV-antibodies raised in chickens and cockatoos. Additionally, two sheep and several psittacine birds were infected with the engineered virus and their antibody response was monitored. It was found that both sheep and birds inoculated with the synthesized virus induced the production of antibodies that recognized native BFDV. These results show that the engineered virus is able to induce an antibody response and those characteristics of the native BFDV have been conserved. The engineered virus has valuable potential future applications in immunisation of parrots, lorikeets and cockatoos. In addition, this experiment has resulted in a large stock of sheep-anti-Beak and Feather Disease Vaccine sera that can be used for diagnostic testing of psittacine birds.

Technical Summary

Full length and C-terminal truncated recombinant beak and feather disease virus (BFDV) capsid proteins were expressed using the bacterial expression system PinPoint™. The full length recombinant protein reacted with sera from naturally immune cockatoo and chicken experimentally inoculated with BFDV. Reactivity proved antigenic epitopes of the BFDV had been conserved in the recombinant protein. The full length recombinant BFDV capsid protein induced an antibody response in two inoculated sheep. Antibodies raised against the recombinant capsid in the sheep recognized native viral inclusions in skin sections from a chronically infected cockatoo by immunohistochemistry. These results confirmed that antigenic epitopes of the BFDV had been conserved in the recombinant protein and are involved in the generation of an antibody response. Haemagglutination inhibition assay demonstrated that some psittacine birds vaccinated with the recombinant protein in conjunction with Freund's incomplete adjuvant produced antibodies that inhibited the haemagglutinating activity of BDFV. This is the first reported evidence of the potential value of a recombinant protein in vaccination and protection of psittacine species against the detrimental effects of BFDV and its future application for preservation of Australian Psittaciforme biodiversity.

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Abbreviations

a.a.	amino acid
ATP	adenosinetriphosphate
BFDV	beak and feather disease virus
bp	base pairs
BSA	bovine serum albumine
CAV	chicken anaemia virus
C-terminus	carboxy terminus
DNA	dioxy ribonucleic acid
ddNTP	dideoxynucleotidetriphosphate
dNTP	deoxynucleotidetriphosphate
DTT	dithiothreitol
ds	double stranded
EDTA	ethylenediaminetetra acetic acid
E. coli	*Escherichia coli*
GIT	gastrointestinal tract
GTP	guanosinetriphosphate
HCl	hydrogen chloride
HI	haemagglutination inhibition
HIU	HI units
HRP	horse radish peroxidase
IHC	immunohistochemistry
IPTG	isopropyl β-D-thiogalactopyranoside
Jca	Jembrana Disease Virus capsid protein
KCl	potassium chloride
KOA	potassium acetate

LB	Luria Bertani
$MgCl_2$	magnesium chloride
NaOH	sodium hydroxyde
NaCl	sodium chloride
nt	nucleotide
N-terminus	aminoterminus
OD	optical density
ORF	open reading frame
ORF C1	open reading frame C1 that codes for BFDV capsid protein
ORF V1	open reading frame V1 that codes for BFDV replication protein
PBFD	psittacine beak and feather disease
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	porcine circovirus
PMSF	phenylmethylsylphonyl fluoride
PolyA	poly adenylation
RCR	rolling circle replication
Rep	replication associated protein
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ss	single stranded
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with tween
TE	Tris EDTA

UV   ultra violet

v/v   volume per volume

w/v   weight per volume

Units

g	relative centrifugation force [=$(\omega^2 \times r) / 980$ cm/s^{-2}]
g/L	grams per litre
g/mL	grams per millilitre
kb	kilo bases
kDa	kilo Dalton
mA	milliampere
mg/mL	milligram per millilitre
mL	millilitres
min	minutes
mM	millimolar
mW	milliwatts
ng	nanograms
rpm	rounds per minute
s	seconds
ρmol	picomoles
µL	microliters
µg/mL	micrograms per millilitre
µM	micromoles
U	units

1   Review of literature on BFDV

This review will consider the current knowledge on beak and feather disease virus (BFDV), its associated disease Psittacine Beak and Feather Disease (PBFD) and other literature relevant to this honours project: Development of Recombinant Beak and Feather Disease Virus Capsid Protein as a Candidate Vaccine for Psittacine Beak and Feather Disease. The review is composed of four sections. Section one will consider the family *Circoviridae* to which BFDV belongs, section two accounts for the pathogenicity, transmission, epidemiology of BFDV and techniques currently available for diagnosing PBFD. Section three accounts for genetics and proteomics of BFDV, and finally section four discusses recombinant protein technology and the application of recombinant proteins.

The nomenclature used in this thesis is that used by Bassami *et al.* (1998) when concerning convention of numbering nucleotide (nt) positions. Nomenclature as described by Niagro *et al.* (1998) is used for labeling open reading frames (ORFs). Finally, taxonomy is by the convention of the International Committee on Taxonomy of Viruses (van Regelmortel *et al.*, 2000).

1.1   Introduction

The most common viral disease of psittacine birds in Australia is PBFD, endemically affecting both wild (Raidal *et al.*, 1993b; McOrist *et al.*, 1984) and captive species (Studdert, 1993). BFDV is listed as a key threatening process for five endangered psittacine species in Australia (Raidal 2004, per comm.) and causes significant problems with breeding of Psittaciformes (Studdert, 1993; Jacobson *et al.*, 1986; Kock *et al.*, 1993).

1.2 The *Circoviridae*

BFDV is a member of the family *Circoviridae*. Viruses of the *Circoviridae* are characterized by a single stranded (ss) circular DNA genome and non-enveloped virions with icosahedral morphology (van Regelmortel *et al.*, 2000). The members of this family appear to be host specific. Family *Circoviridae* currently holds two genuses, *circovirus* and *gyrovirus*. BFDV, porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2) are gathered in the genus *circovirus*. Chicken anaemia virus (CAV) is the only member of the genus *gyrovirus* (van Regelmortel *et al.*, 2000). CAV is classed in a separate genus within the *Circoviridae* due to some features significantly different to the other circoviruses, including a single polycistronic message encoding all viral proteins, lack of a stemloop structure, and not possessing an ambisense genome. There are no common antigens reported between these animal circoviruses (van Regelmortel *et al.*, 2000). Circoviruses cause a wide range of disease in different animal hosts. CAV causes transient anemia and immunosuppression in young chicks (van Regelmortel *et al.*, 2000). PCV1 was first discovered in the continuous porcine kidney cell line PK-15, however no disease has been associated with the virus (Tischer *et al.*, 1986). PCV2 in comparison is believed to be associated with post weaning multisystemic wasting syndrome (van Regelmortel *et al.*, 2000).

1.3 Clinical presentation and pathology of PBFD

PBFD is most often seen in young captive birds under the age of three years. The condition is apparently irreversible, and lasts from several months to a year or even several years (Pass and Perry, 1984; Jacobson *et al.*, 1986). PBFD occurs as a chronic or acute condition. Symmetrical loss and dystrophy of contour, crest, tail and down feathers are typically seen in chronic PBFD (Figure 1-1) and mainly occurs in older birds. Wing feathers may also less commonly be affected. The abnormal feathers may

present with at least one of the following characteristics: retained feather sheath, feather sheath filled with blood, short clubbed feathers, curled and deformed feathers, feathers with circumferential constriction, and stress lines in the vane (Pass and Perry, 1984; McOrist *et al.*, 1984; Jergens *et al.*, 1988; Jacobson *et al.*, 1986). Feather lesions are only seen in feathers developed after infection with BFDV (Wylie, 1991). Therefore birds with readily developed feathers may not exhibit feather lesions until the bird has moulted.

Figure 1-1. Two sulphur crested cockatoos chronically infected with BFDV displaying gross clinical signs of feather loss.

The beak may exhibit changes in colour, progressive elongation, palatine necrosis, and longitudial and transverse fractures (Figure 1-2) (Pass and Perry, 1984; Jergens *et al.*, 1988; McOrist *et al.*, 1984). Beak and feather pathology are due to epidermal cell necrosis, epidermal hyperplasia and hyperkeratosis (Pass and Perry, 1984; McOrist *et al.*, 1984).

Figure 1-2. A galah chronically infected with BFDV displaying gross clinical signs of beak fracture.

Acute disease, most commonly observed in fledgling and immature birds, differs distinctly from the chronic form as it does not usually manifest with beak and feather abnormalities, however is characterised by lethargy, depression, diarrhoea and often results in death (Raidal, 1994; Ritchie *et al.*, 1989a). Severe leukopaenia, anaemia, or pancytopaenia and liver necrosis, have been reported in birds diagnosed with peracute PBFD. These birds, as with acutely infected birds, lacked feather and beak abnormalities (Schoemaker *et al.*, 2000). Route of infection, virus titre, age, condition of the bird at the time of infection and species have been postulated as factors determining whether the disease has a chronic or an acute outcome (Ritchie *et al.*, 1989a; Wylie and Pass, 1987; Raidal and Cross, 1995).

The target organ of BFDV has been shown to be the epithelium, primary site of virus replication most likely being the bursa of Fabricius, but may also occur in internal organs. BFDV antigens have also been demonstrated in liver, kidneys, thymus, bone marrow and other internal organs (Pass and Perry, 1984). Evidence by Raidal *et al*. (1993a) suggests that the liver is an important site for BFDV replication in both acute and chronic disease. Experimental infection of nestling sulphur-crested cockatoos by Raidal and Cross (1995) and studies on African grey parrots (*Psittacus erithacus*

erithacus) by Schoemaker *et al.* (2000) showed that BFDV infections may cause necrotizing hepatitis. Additionally, the latter authors report findings of severe leukopaenia in such parrots naturally infected with BFDV.

1.4 Immunology associated with PBFD

Damaged lymphoid tissue and immune suppression occurs typically with BFDV-infection. Populations of both helper (CD4$^+$) and cytotoxic (CD8$^+$) T cells are depleted, due to the virus targeting precursor T cells. This results in a predisposition to secondary bacterial and fungal infections, which may result in mortality (Schoemaker *et al.*, 2000; Latimer *et al.*, 1992). Wylie and Pass (1987) found occasional infiltration of heterophils and lymphocytes into the pulp of feathers during experimental reproduction of PBFD using budgerigars. These findings have been further supported by Jacobson *et al.* (1986), indicating that feather lesions and abnormalities are associated with the immune response.

1.5 Transmission of BFDV

Feather dust has been proposed as a major vehicle of transmission, due to the high amount of viral inclusion bodies that may be found in the follicular and feather epithelium of BFDV positive birds (Pass and Perry, 1984; McOrist *et al.*, 1984; Ritchie *et al.*, 1991). Necrotic epithelial cells in the outer layers of hyperkeratotic feather sheaths contain intracytoplasmic viral inclusion bodies (Pass and Perry, 1984), supporting the theory of feather dust as a vehicle for transmission. One likely mode of transmission may occur by ingestion during preening activities (Ritchie *et al.*, 1991). The second possible mode of transmission is the faecal-oral route. The gastrointestinal tract (GIT) has been postulated as a site of viral replication, due to findings of viral-induced inclusion bodies in several locations of the GIT, and therefore explains findings

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of BFDV in the faeces of infected birds. Thus transmission is likely to occur via the faecal-oral route (Ritchie *et al.*, 1991; Raidal and Cross, 1995). Thirdly, crop washings have also been shown to contain BFDV. Even though the concentration of virus was low, it is possible that the virus can be transmitted from adults to neonates during feeding activities involving regurgitation of food and crop epithelium (Gerlach, 1994). Supporting this theory, vertical transmission of BFDV has been demonstrated with an infected hen, where the chicks consistently developed lesions associated with PBFD (Gerlach, 1994). A fourth possible mode of transmission is, according to Raidal (1994), direct deposition of contaminated material into the cloaca of nestling birds. Nestlings have weak legs and poor balance, and are forced to sit tripod like on their legs and abdomen. When they defecate, nestlings slide their cloaca over nesting material, thus possibly contracting infections.

1.6   Epidemiology of BFDV

Between 1887 and 1888 Ashby (1907) observed a decline in the population of *Psephotus* parrots in the Adelaide Hills, and described "feathering abnormalities" causing impaired flight as the reason for this decline. This first reported outbreak of feather disease in wild parrots was probably due to BFDV (Raidal *et al.*, 1993b). PBFD occurs endemically in both captive and wild psittacine species (Raidal *et al.*, 1993b; Raidal, 1994) and has been reported in both old and new world captive psittacine species (Ritchie *et al.*, 1991; Kock *et al.*, 1993; Raidal, 1995). Histological and clinical observations suggesting PBFD have been described in 42 psittacine species (Gerlach, 1994). Approximately 30 and perhaps all 50 species of Australian psittacine birds, both captive and wild, are affected by PBFD (Studdert, 1993). The disease is largely found in sulphur-crested cockatoos (*Cacatua galerita*). Based on gross pathology and histology, McOrist *et al.* (1984) found that several flocks of sulphur-crested cockatoos in Victoria

had a 10-20% incidence of PBFD. In some wild populations, seroprevalence as high as 94% has been reported, however the exact number of affected birds in a flock is hard to determine due to an unknown number of birds affected by PBFD dying in the nest (Raidal *et al.*, 1993b). PBFD has also been demonstrated in galahs (*Cacatua roseicapilla*), little corella (*Cacatua sanguinea*), Major Mitchell's cockatoo (*Cacatua leadbeateri*), lovebirds (*Agapornis spp.*), budgerigars (*Melopsittacus undulatus*), African grey parrot (*Psitticus erithacus erithacus*), short-billed corella (*Cacatua sanguinea*), eastern long-billed corella (*Cacatua tenuirostris*), blue bonnet (*Psephotus haematogaster*) rainbow lorikeet (*Trichoglossus haematodus*) and other psittacine species (Schoemaker *et al.*, 2000; Raidal *et al.*, 1993b; Ritchie *et al.*, 2003). PBFD is the most commonly encountered disease in captive and wild psittacine birds in Australia, where it currently threatens five species [(orange-bellied parrot (*Neophema chrysogaster*), swift parrot (*Lathamus discolour*), Norfolk Island green parrot (*Cyanoramphus neozelandiae cookie*), superb parrot (*Polytelis swainsonii)* and Eastern regent parrot (*Polytelis anthopeplus monarchoides*)] (Raidal 2004, pers comm).

1.7   Diagnosis of PBFD

The primary procedure for diagnosis of PBFD is clinical examination on the basis of gross pathology. Diagnosis based on observation of clinical presentation is not in itsself sufficient for an accurate diagnosis, as many practitioners fail to detect pathognomonic feather lesions in early clinical cases (Raidal *et al.*, 1993c) and clinical signs may be confused with polyomavirus infections and other conditions (reviewed in Riddoch (1996)). Observing epidermal hyperplasia and hyperkeratosis of feather follicles using histopathological examination of feather follicles (Pass and Perry, 1984) may be used as a routine measurement, but requires biopsy of feather follicles, causing injury to the bird. Histopathology is of also limited use when testing flocks (Raidal *et al.*, 1993c).

Polymerase chain reaction (PCR) (Ypelaar *et al.*, 1999) and haemagglutination (Raidal and Cross, 1994b) will detect BFDV nucleic acid and antigen, respectively, and a haemaglutination inhibition assay (HI) (Raidal *et al.*, 1993c) has been developed for the detection of BFDV antibody. Diagnostic techniques such as immunohistochemistry (IHC), transmission electronmicroscopy, DNA dot-blot hybridization (Raidal *et al.*, 1993c) *in situ* hybridisation (Ramis *et al.*, 1994) and feather enzyme immunoassay (Raidal, 1994) have been described.

1.8   Vaccination and control of PBFD

Raidal and Cross (1994a) proved vaccination to be an effective means of controlling PBFD. This experiment used an aviary flock of 77 *Agapornis spp* and vaccine produced from the feathers of PBFD-affected sulphur-crested cockatoos. Subsequent generations after vaccination did not develop disease either (Raidal and Cross, 1994a). A double-oil emulsion killed vaccine, produced by Raidal *et al.* (1993a) was successful at protecting nestling galahs. These experimental findings indicate that PBFD can be controlled in individual, as well as flocks of psittacine species by vaccination.

Attempts at culturing BFDV in numerous culture systems have been unsuccessful (Pass and Perry, 1985). Thus development of a recombinant protein vaccine would be a useful tool in the control of this endemic disease in wild and aviary flocks as well as pet psittacine birds.

1.9   Genetic variation in BFDV

Initially Ritchie *et al.* (1989b) determined that the genome of BFDV was a single ssDNA between 1.7 to 1.9 kb in size. Bassami *et al.* (1998) determined a more accurate size of 1993 nt. Further research by Bassami *et al.* (2001) compared eight new isolates

of Australian BFDV, to the isolate (BFDV-AUS) described by Bassami *et al.* (1998) and the BFDV-USA isolate derived from pooled BFDV reported in the USA by Niagro *et al.* (1998). The eight new isolates ranged between 1992 and 2018nt in genomic size and had an overall nt identity between 84 and 97% compared to the BFDV-AUS isolate. The nt sequence identity of ORF C1 in the eight Australian isolates studied by Bassami *et al.* (2001) varied from 80 to 99% when compared to the BFDV-AUS isolate. Further, of the seven ORFs detected in the BFDV-AUS isolate, only three (ORF V1, ORF C1 and ORF5) were consistently detected in all 10 isolates compared. Point mutations and several deletions and insertions from 1 to 17 nt in size in both coding and non-coding regions accounted for the variation in these isolates.

When considering location of ORFs, hairpin structure, nonanucleotide motif, the three motifs in the rep protein required for rolling circle replication (RCR), the P-loop motif and poly adenylation (polyA) signal downstream of ORF V1 and ORF C1, and the octanucleotide motif downstream of the hairpin structure, all these isolates were homologous, except for in one isolate from a blue bonnet (*Psephotus haematogaster*) in Western Australia, where there was a base substitution in the second octanucleotide motif.

1.9.1   BFDV genome

Further discussion of BFDV genome related topics will be based on the BFDV-AUS isolate. BFDV has an ambisense genome, with a size of 1993 nt (Bassami *et al.*, 1998; Niagro *et al.*, 1998). Seven ORFs, three on the viral strand and four on the complementary strand (Figure 1-3) tentatively encoding proteins of >8.7kDa have been reported (Bassami *et al.*, 1998). Flanked by ORF C1 and V1 in the replicative form, the genome of BFDV has a nt motif with the capacity to form a stem loop structure at nt position 1976-1993 and 1-12 (Figure 1-4). The apex of the BFDV stemloop is the

nonanucleotide motif 5'-TAGTATTAC-3'. This motif is highly conserved within the *Circoviridae* and other circular ssDNA viruses (Table 1-1). Immediately downstream of the stemloop structure is an octanuclotide repeat sequence 5'-GGGCACCG-3' (Figure 1-4) (Bassami *et al.*, 1998).
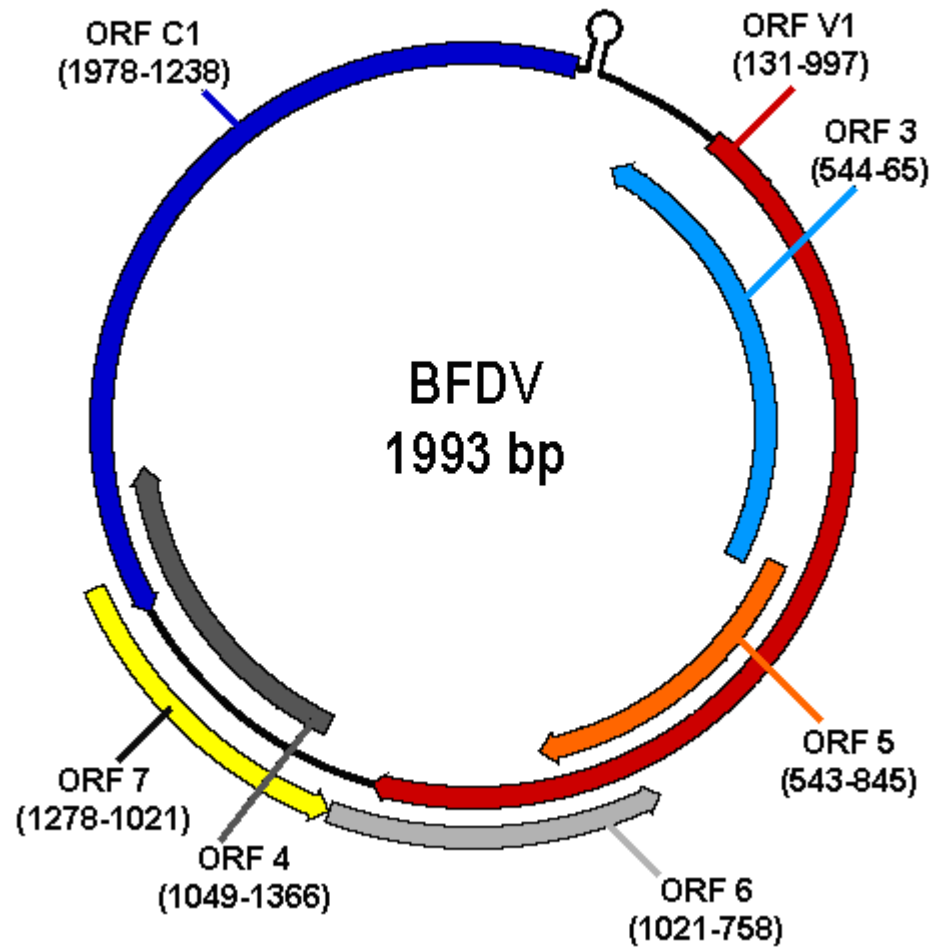
Figure 1-3. Schematic diagram of BFDV double stranded replicative form displaying location of ORFs as outlined Bassami et al. (1998).
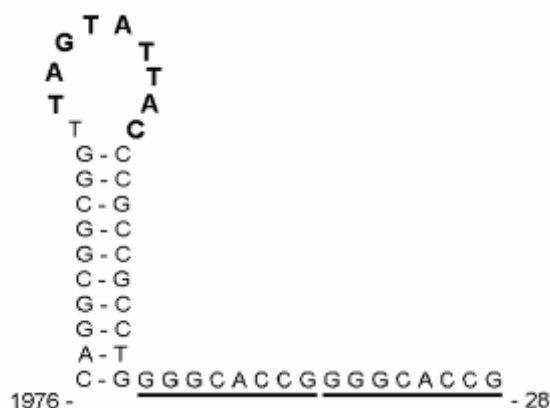
Figure 1-4. The potential stemloop structure of BFDV. In bold is the nonanucleotide motif and underlined is the octanucleotide repeat sequence. Adopted from Bassami (1998).

Table 1-1. Sequence alignment of nonanuclotide motif of BFDV (accession No. AF080560) (Bassami *et al.*, 1998), PCV (accession No. U49186) (Meehan *et al.*, 1997), CAV (accession No. D10068) and the geminiviruses: Tomato golden mosaic virus (TGMV), squash leaf curl virus (SLCV) and dicot-infecting geminiviruses (DIG) (Arguello-Astorga *et al.*, 1994). *, indicates conserved nt within the motifs.

Virus species	Nt sequence (5' - 3')
BFDV	TAGTATTAC
CAV	TACTATTCC
PCV	TAGTATTAC
TGMV	TAATATTAC
SLCV	TAATATTAC
DIG	TAATATTAC
	* * * ** * * *

The viral strand has two potential TATA boxes, TATA at nt 86-89 and TATAAAA at nt 680-686 upstream of the start codon for ORF V1, and two polyA signals downstream of the stopcodon for ORF V1 at nt 1019-1024 and 1196-1201. The complementary strand of the replicative form features a polyA signal, AATAAA at nt 758-763, only one nt downstream of ORF C1 (Bassami *et al.*, 1998). The function of these TATA boxes was not determined or hypothesized.

1.9.2   *ORFs and protein products*

1.9.2.1   *ORF C1*

The 741 nt ORF C1 is located on the complementary strand of the double stranded (ds) replicative form (nt position 1978-1238). This ORF encodes a potential 28.9 kDa protein product hypothesized to be the capsid protein (Bassami *et al.*, 1998). The predicted protein product of the equivalent ORF in PCV was identified on the complementary strand, and shared 29.1% amino acid (a.a.) sequence identity with BFDV. The ORF starts within or close to the stemloop structure of the respective viruses, indicating similarity in function (Bassami *et al.*, 1998). BFDV and PCV ORF C1 predicted protein products feature a highly conserved 14 a.a. sequence close to the C-terminus with an adjacent myristylation site (Niagro *et al.*, 1998). The highly conserved nature of these features pleads their vast necessity for protein function. PCV2 ORF C1 cloned into baculovirus expression vector by Nawagitgul *et al.* (2000) resulted in expression of a 30 kDa product. Similar results were achieved with purified virus particles. Electron microscopic viewing of the recombinant protein detected a self-assembled protein forming capsid-like particles. Similar results have been reported by Liu *et al.* (2001)**.** These reports are experimental proof for PCV2 ORF C1 encoding a capsid protein, and strengthens the assumption that BFDV ORF C1 also encodes a

capsid protein when correlating these results with the 29.1% sequence identity found between ORF C1 of BFDV and PCV reported by Bassami *et al.* (1998).

1.9.2.2   ORF V1

ORF V1 is 867nt long, located on the viral strand and was predicted to encode a 33.3 kDa protein product by Bassami *et al.* (1998). This putative protein features all sequence motifs associated with proteins involved in RCR. A P-loop motif has been identified in the reading frame (Niagro *et al.*, 1998). P-loop motifs and ATP/GTP binding motifs are found in proteins with helicase activity (Hodgeman, 1988). A potential pyrophosphatase domain is found in the predicted a.a. sequence of this protein (Niagro *et al.*, 1998). Bassami *et al.* (1998) demonstrated a high a.a. similarity (45.6%) between the product of BFDV ORF V1 and the replication-associated protein of PCV, subterranean clover stunt virus and faba bean necrotic yellows virus. Thus, it is likely that ORF V1 of BFDV also encodes a replication-associated protein.

1.10 BFDV replication

BFDV has a very small genome and a limited protein expression capacity; therefore DNA replication must be heavily dependant on the host cell machinery (Todd, 2000). No reports have been published on the exact mechanism of replication in BFDV. Circular ssDNA viruses replicate using a mechanism known as RCR (Gutierrez *et al.*, 2004; Gronenborn, 2004). The predicted replication-associated protein of BFDV, possesses all motifs involved in RCR (Niagro *et al.*, 1998), thus it is most likely that BFDV also replicates via this mechanism to produce progeny virus. In addition, the stem loop structure and nonanuclotide motif previously described are common features of ssDNA viruses that replicate via RCR. Geminiviruses are ssDNA viruses proven to replicate via RCR. They feature the nonanucleotide motif with high sequence homology

to BFDV and PCV (Table 1-1) and, as with BFDV, have a short inverted repeat sequence flanking the nonanucleotide motif (Arguello-Astorga *et al.*, 1994; Bassami *et al.*, 1998). CAV, even though not possessing the stemloop structure, has a very similar nonanucloutide motif to BFDV. ORF V1 of PCV, which is closely related to BFDV, encodes a putative replication-associated protein. This protein carries all the sequence motifs commonly found and required for RCR, and additionally a P-loop motif (Niagro *et al.*, 1998). Although experimental proof is not available, these findings in BFDV and its similarities to other circular ssDNA viruses, suggest that BFDV replicates via RCR.

1.11 Recombinant protein technology

1.11.1 Recombinant protein expression systems

Many systems have been developed for production of recombinant proteins, including bacterial, yeast, mammalian cell, insect cell and cell free systems. Today's knowledge of *E. coli* genetics, molecular biology and biochemistry far exceeds that of any other organism. Bacteria can be grown and genetically manipulated in an easy and inexpensive manner, and many foreign proteins are expressed at a high level (Sambrook and Russel, 2001). Due to these characteristics, bacterial systems and their phages are the preferred choice of laboratories engaging in development of recombinant proteins. Conversely, use of bacterial systems for expression of biologically active proteins may incur problems, due to the absence of posttranslational modification in these systems. Additionally, the foreign proteins may become tied up in inclusion bodies, making purification of the protein intricate (Coligan *et al.*, 2002; Sambrook and Russel, 2001).

Problems associated with bacterial recombinant protein expression can be overcome with the use of eukaryotic systems including mammalian and insect cell expression systems. For example baculovirus expression systems utilize insect cells, which are

eukaryotic cells. Therefore protein modifications, processing and transport systems are similar to those found in higher eukaryotes. Large amounts of recombinant protein can be relatively easy produced in baculovirus systems by using a helper independent virus that can be propagated to high titres in insect cells adapted for growth in suspension culture (Coligan *et al.*, 2002). In contrast to bacterial systems, most of this over expressed protein will stay soluble in the cells. Thus baculovirus is increasingly becoming a popular choice in recombinant protein technology (Coligan *et al.*, 2002). The majority of protein expressed in this system occurs during acute lytic infection of the insect cells, resulting in addition to more effort and expertise, a requirement for constant generation of new virus stocks and cells (Sambrook and Russel, 2001).

Yeast systems, also eukaryotic, have for a long time been utilised for expressing recombinant proteins, however they have been found to be vastly inefficient for protein expression, producing low quantities of protein and are therefore not ideal for experiments where the aim is to produce significant recombinant protein for use in, for example, vaccination (Sambrook and Russel, 2001).

For this honours project, an *E. coli* based system was chosen. The reason for this is the ease with which recombinant bacteria can be made, use of inducible promoters and fusion tags allowing for controlled expression and purification of the protein, and finally the low cost of using an *E. coli* based system.

1.11.2 *Application of recombinant proteins*

Recombinant proteins have several uses. Haemaglutination inhibition (HI) is a specific and sensitive technique used for detection of circulating antibodies in animals, indicating whether the animal has previously been challenged with an infection of a haemagglutinating virus. The technique is a standard serological test for many

haemagglutinating viruses. BFDV is a haemagglutinating virus (Raidal and Cross, 1994b) and a HI technique has been developed (Raidal *et al.*, 1993c). However, this technique requires access to native virus, and thus limits the use of this technique for wide spread diagnosis of BFDV infection. A recombinant BFDV capsid protein would allow for more extensive use of this technique.

Enzyme-linked immunosorbent assay (ELISA) is another technique used for detection of circulating antibodies and is a valuable tool in disease diagnosis (Roitt *et al.*, 2001). Recently, Johne *et al.* (2004) developed an ELISA and an immunoblotting technique successful in detecting antibodies to BFDV in psittacine sera, using a truncated recombinant BFDV capsid protein expressed in *E. coli*. ELISA based on Baculovirus-expressed PCV-2 capsid protein has also been described (Liu *et al.*, 2004).

Recombinant proteins may also be applied as vaccines for control of viral, bacterial, parasitic and other diseases. The truncated recombinant BFDV capsid protein developed by Johne *et al.* (2004) was shown to be successful in raising an antibody response in a chicken. Immune response to recombinant hemagglutinin-neuraminidase glycoprotein of *Perste des petits ruminants* virus in goats has been reported (Sinnathamby *et al.*, 2001). The cathepsin B-like protein of juvenile *Fasciola hepatica* has been cloned and expressed in yeast and shown to be antigenic in vaccinated rats (Law *et al.*, 2003). Vaccination against *Fasciola hepatica* resulting in significant protection using a recombinant antigen, has been reported (Almeida *et al.*, 2003).

1.12 Hypothesis

A recombinant BFDV capsid protein expressed using a bacterial system is antigenic, causing the host to respond by producing antibodies that would recognize both the recombinant protein and native virus particles.

1.13 Aims

The aims of this honours project were to:

1) Produce a recombinant BFDV capsid protein, using a bacterial expression system.

2) Determine whether the recombinant protein is recognised by sera from naturally infected and challenged bird species.

3) Determine whether the protein induces an antibody response in sheep and whether these antibodies will recognize native virus.

4) Determine whether the protein induces an antibody response in psittacine birds.

2   Materials and Methods

2.1   Viral DNA

Feathers or blood samples known to contain BFDV were provided by the Diagnostic Pathology Service of Murdoch University Veterinary Hospital. The QIAamp DNA Blood Mini kit (QIAGEN, Australia) was used to extract BFDV DNA according to the directions provided by the manufacturer as follows. Twenty microliters of proteinase K was pipetted into the bottom of a 1.5 mL microcentrifuge tube and 200 μL whole blood added. Two hundred microliters of buffer AL was further added and mixed by pulse-vortexing for 15 s. The sample was then incubated for 10 min at 56°C and then microcentrifuged briefly to remove drops from the inside of the lid. Two hundred microliters of 100% ethanol was added, mixed by 15 s pulse-vortexing and briefly centrifuged again. The mixture was then transferred to a QIAamp spin column inserted in a 2 mL collection tube and centrifuged at 6,000 *g* for 1 min. The spin column was transferred to a new collection tube and centrifuged for 1 min at 6,000 *g*. Five hundred microliters of buffer AW1 was added to the tube and centrifuged as before. The spin column was then transferred to a clean collection tube, 500 μL buffer AW2 added and centrifuged at 20,800 *g* for 3 min. The spin column was transferred to a new 1.5 mL microcentrifuge tube, 200 μL buffer AE added, incubated at room temperature for 1 min and finally centrifuged for 1 min at 6,000 *g*.

2.2   Cells used for expression of recombinant proteins

High efficiency JM109 competent *Escherichia coli* cells (Stratagene, Australia) were used for transformation and expression of recombinant BFDV capsid protein. This *E.*

coli strain is of the genotype *e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17*(rK- mK+)

supE44 relA1 Δ(lac-proAB) [F' *traD36 proAB lacI^qZΔM15]*.

2.3   Amplification of BFDV ORF C1

PCR was used to amplify BFDV ORF C1 from extracted viral DNA. All reactions were

performed using thermal cyclers: Applied Biosystems GeneAmp PCR system 2400,

BioRad MyCycler or the Eppendorf Mastercycler Gradient thermal cycler in 0.2 mL

microcentifuge tubes (Sarstedt, Germany). Specific primers were designed using

computer programs Amplify for Analysing PCR Experiments (Engels, 1992) and

Primer Express (version 1.0, ABI Prism, Applied Biosystems) based on the BFDV-AUS

isolate (accession number: AF080560) and analysed using blastn (Altschul *et al.*, 1997)

in BioManager by ANGIS (http://www.angis.org.au). The region of ORF C1 for

producing full length 5' truncated and 3' truncated recombinants was chosen on the

basis of published sequence data (accession number: AF080560) and predicted

antigenicity and hydrophilicity charts using the algorithms Antigenic index and

Kyle/Doolittle hydrophilicity in MacVector (version 6.5.1, Oxford Molecular Group,

Madison, WI, USA). These predictions can be viewed in Figure 2-1. The latter would

result in expression of a carboxy-terminus (C-terminus) truncated recombinant capsid

protein. The design of 5' truncated recombinants was based on predicted hydrophilic

regions within the protein and would express an aminoterminus (N-terminus) truncated

recombinant capsid protein. The three regions of ORF C1 were amplified using: primers

Forward 1 and Reverse 1 (primer set A) for full length; Forward 1 and Reverse 2

(primer set B) for 3' truncated and primers Forward 2 and Reverse 1 (primer set C) for

5' truncated amplification of the ORF C1. Position and orientation of primers can be

seen in Figure 2-2 and primer details can be seen in Table 2-1.
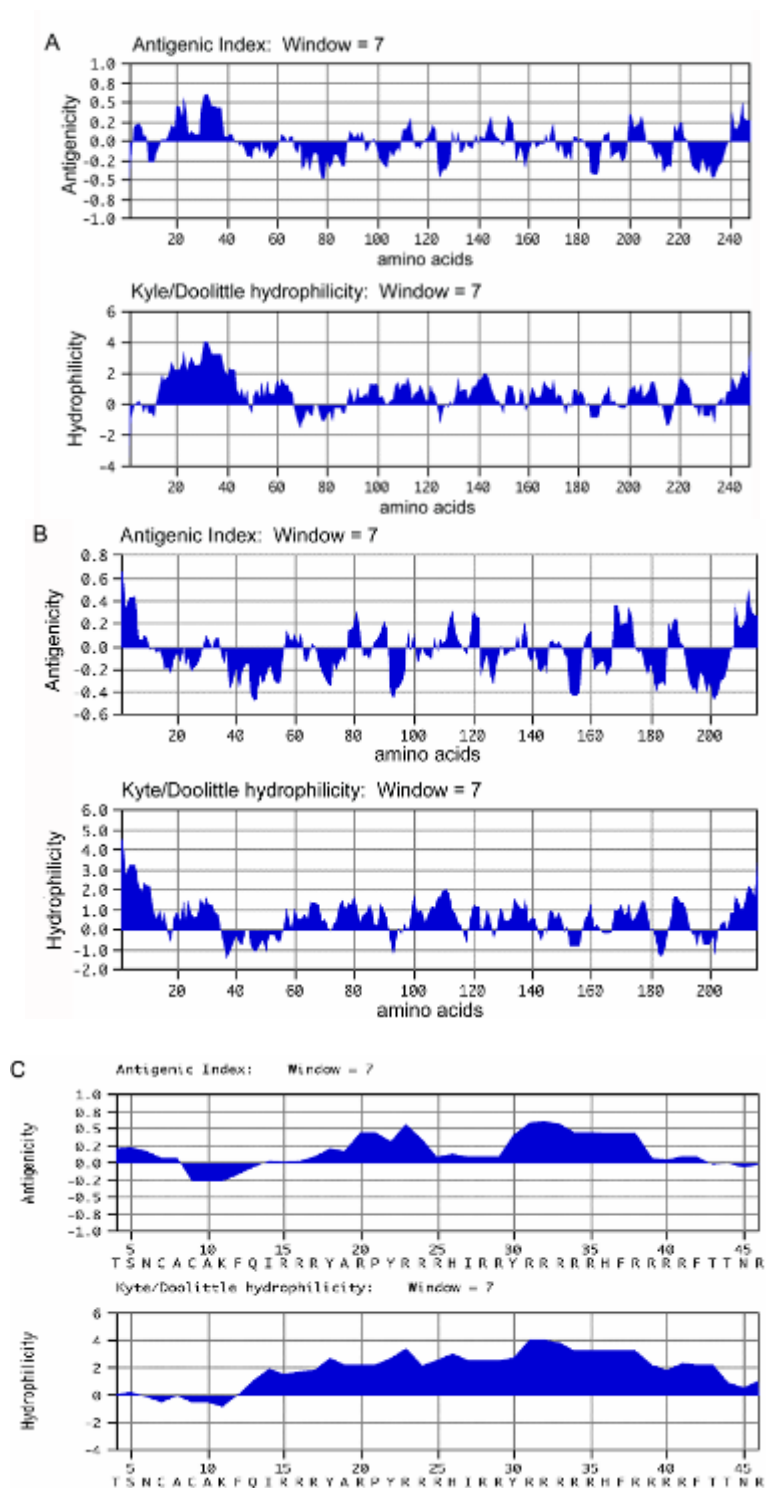
Figure 2-1. Antigenicity and hydrophilicity charts of A) full length BFDV capsid protein, B) region chosen for 5' truncated BFDV capsid protein and C) region chosen for 3' truncated BFDV capsid protein.
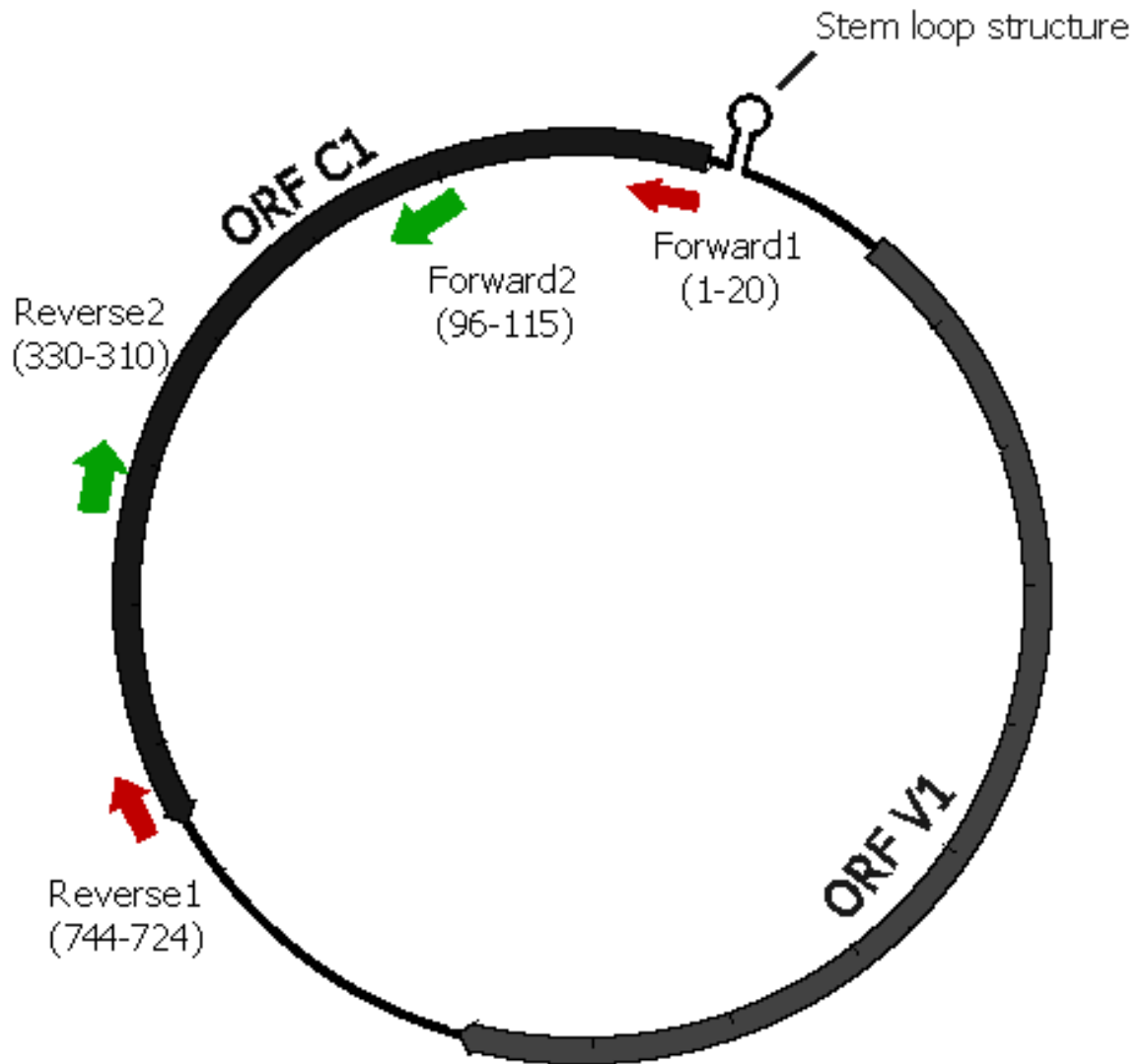
Figure 2-2. Position and orientation of primers in the BFDV genome (ds replicative form) used for full length and truncated ORF C1 amplification.

Table 2-1. Primer sequences used to amplify ORF C1 full length and truncated fragments of the BFDV genome. The binding position of the primer within ORF C1 is given (nt).

Primer	Primer sequence	nt position in ORF C1 (primers: Forward 1 and 2, and Reverse 1 and 2) and nt position in vector (primers: SP6 and pinseq)
Forward 1	5'cat gtg ggg cac ctc taa ct3'	1-20
Reverse 1	5'tta agt gct ggg att gtt agg ggc3'	744-724
Forward 2	5'ccg acg tag gca ctt ccg ca3'	96-115
Reverse 2	5'ggg cct cat ttc cat ttt3'	330-310
SP6	5' tat tta ggt gac act ata g 3'	451-468
Pinseq	5' cgt gac gcg gtg cag ggc g 3'	325-343

Thermal cycle conditions were optimised for optimal primer annealing temperature using an annealing temperature gradient as described below in section 2.3.1. The PCR conditions: magnesium chloride concentration, primer concentration and dNTP concentration, were optimised according to the Taguchi method applied to PCR described by (Cobb and Clarkson, 1994) and outlined in section 2.3.2.

2.3.1   *Optimization of annealing temperature*

A basic PCR mixture with excess primers and dNTPs was utilised to determine the annealing temperature range that could be used for amplification. PCR reactions were carried out in a total volume of 30 μL and consisted of 0.7 U Expand High Fidelity DNA polymerase (Roche), 1× polymerisation buffer (composition not supplied, Roche), 2.083 mM MgCl$_2$ (Roche), 300-400 ng DNA template, 0.083 mM dNTPs, 25-35 ρmol

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of each primer and made up to 30 μL with Ultra pure water (Fisher Biotec, Australia). Thermal cycle protocol for temperature optimisation is outlined in Table 2-2.

Theoretical optimal annealing temperature for each primer was calculated by the formula $((g+C)\times4)+((A+T)\times2)$. The annealing temperature calculated for primers Forward 1, Reverse 1 and Forward 2 appeared to be excessively high, thus a closer to normal temperature of 56°C was chosen as the mean temp. Additionally, the calculated annealing temperatures for the two primers in each pair differed significantly, thus two annealing temperatures were used in the thermal cycle to accommodate these differences. Further, the optimal annealing temperatures were found by using a temperature gradient, deviating ±5°C from the theoretical temperature determined, on an Eppendorf Mastercycler Gradient thermal cycler. Finally, the annealing temperature giving the highest yield of amplification was determined to be 56.4°C and 44.7°C for primer sets A and C, and 53.7°C and 42°C for primer set B by examining the fluorescence strength of fragments visualised by electrophoresis as described under section 2.4.

Table 2-2. Thermal cycling protocol used in optimisation of annealing temperature of the PCR.

Stage	Temperature (°C)	Time (min)	Cycles
1	95	5	1
2	95	0.5	
	56 ± 5	0.5	30
	44 ± 5	0.5	
	72	1	
3	72	10	1
4	15	HOLD	

2.3.2 *Optimization of magnesium chloride, primer and dNTP concentration for PCR amplification of BFDV ORF C1*

Reactions were set up at a final volume of 30 μL, containing 0.7 U of Taq DNA polymerase (Roche), 1×polymerisation buffer (composition not supplied, Roche) and 300-400 ng DNA template. Three different concentrations of $MgCl_2$, primer and dNTP (Table 2-3) were chosen and arranged in an orthogonal array (Table 2-4) to meet the requirements of the Taguchi method for PCR optimisation. In an orthogonal array, each concentration (A, B or C) occurs an equal number of times between each row, thus less reactions are required to determine the effects of each variable ($MgCl_2$, primer and dNTP) (Cobb and Clarkson, 1994). The thermal cycle condition was carried out using the protocol outlined in Table 2-2 using optimal annealing temperatures as determined.

Table 2-3. Concentrations used for optimisation of PCR magnesium chloride, primer and dNTP concentration. Concentrations of each reagent were used in combinations as seen in Table 2-4.

	Concentrations		
Reagent	*A*	*B*	*C*
dNTPs	0.033 mM	0.067 mM	0.1 mM
MgCl$_2$	0.417 mM	1.25 mM	2.08 mM
Primer	25-35 ρmole	31-44 ρmole	38-52 ρmole

Table 2-4. Orthogonal array used for optimisation of PCR magnesium chloride, primer and dNTP concentration.

	Reaction								
Reagent	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
dNTPs	A	A	A	B	B	B	C	C	C
MgCl$_2$	A	B	C	A	B	C	A	B	C
Primers	A	C	B	C	A	B	C	A	B

Optimal dNTP, MgCl$_2$, and primer concentrations were determined to be 0.1 mM dNTPs, 1.25 mM MgCl$_2$ and 31-44 ρmole primers for primer set A; 0.067 mM dNTPs, 2.08 mM MgCl$_2$ and 25-35 ρmole primer for primer set B; and 0.033 mM dNTPs, 1.25 mM MgCl$_2$ and 38-52 ρmole primers for primer set C by examining the fluorescence strength of fragments visualised by electrophoresis as described under section 2.4.

2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using horizontal gels of 0.8 or 1.0% (w/v) agarose (Progen, Australia), TAE buffer (40 mM Tris-HCl; 20 mM glacial acetic acid; 2 mM EDTA; pH 7.0) and 10 μg/mL ethidium bromide (Sigma, Australia). PCR products and DNA were loaded into the agarose gel and electrophoresed for 30 min at 90 Volts in TAE buffer using a Minisub DNA cell (BioRad, Australia). Fragments were visualized

by UV illumination at 260 nm with a UVP transilluminator (BioRad, Australia) or the Gel Doc-1000 system and Molecular Analyst Software version 1.4 (BioRad, Australia). The size of the separated DNA fragments was determined by comparing them with a 1 kb DNA ladder (Promega, USA), 100 bp DNA ladder (Invitrogen, Australia) or a 1 kb plus DNA ladder (Invitrogen, Australia).

2.5   Ligation of PCR products into expression vector

The linear Promega pPinPoint™ Xa-1 T-vector was used to construct the recombinant vector for expressing recombinant BFDV capsid proteins. This vector has a coding region for Beta-lactamase (AmpR), multiple cloning region, biotin purification tag coding region, taq promoter, SP6 RNA polymerase promoter and a T7 RNA polymerase promoter (Figure 2-3). This vector is linear and deoxythymidine tailed, and therefore does not require restriction digest before ligation, only Taq polymerase amplification of insert resulting in the appropriate deoxyadenine overhangs.
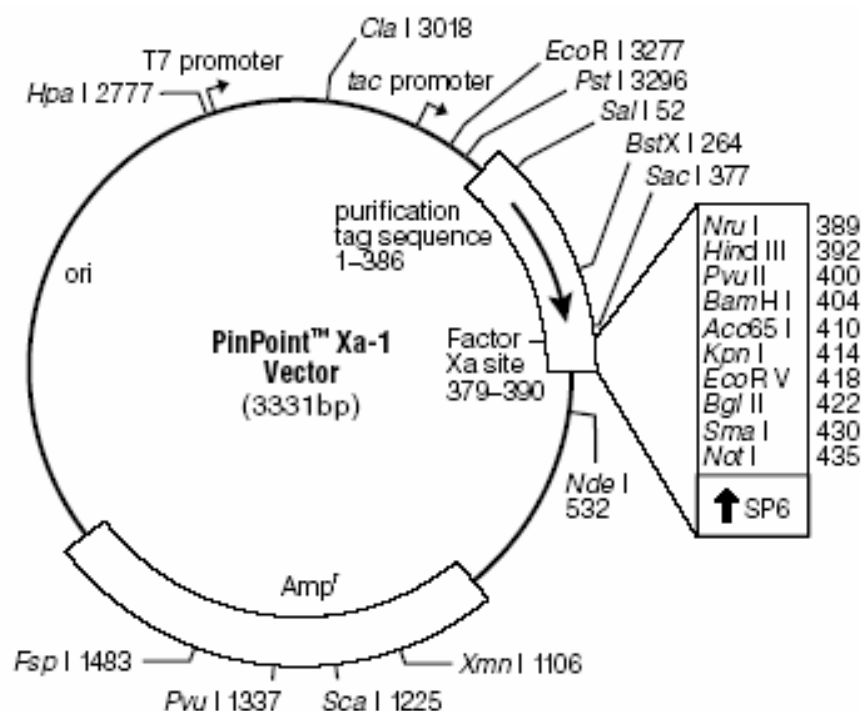
Figure 2-3. The Promega pPinPoint™ Xa-1 vector circle map and sequence reference points. The T-vector is produced by digesting the Promega pPinPoint™ Xa-1 vector with *Eco*R V and adding a 3' terminal deoxythimidine to both ends (Promega, 2000).

One ligation reaction was set up for each amplicon and the PCR product was not purified prior to ligation. Ligation reactions were set up in 0.65 mL microcentrifuge tubes with 12.5 ng vector, 1.0 U T4 DNA ligase, T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/mL BSA; pH 7.8; New England BioLabs, Australia), 0.25 µL PCR reaction product and made to a final volume of 10 µL with distilled water. Reactions were incubated in a 15°C water bath overnight.

2.6 Transformation of JM109 competent cells with recombinant pPinPoint™ Xa-1 T-vector

Two microlitres of ligation reaction was transferred to a 1.5 mL microcentrifuge tube (Sarstedt, Germany) and centrifuged 20,800 *g* for 4 to 5 s to collect ligation mix at the

bottom. Chemically competent *E.coli* JM109 were removed from -80°C storage and thawed on ice. Fifty microlitres of cell suspension was added to the tube containing the ligation reaction and the contents of each tube mixed by gentle flicking. Tubes were then incubated on ice for 30 min prior to heat shock transformation of the JM109 *E.coli* for 50 s in a 42°C water bath and then incubated on ice for 2 min. Nine hundred and fifty microliters of SOC medium (0.02 g/mL Bacto-tryptone, 0.005 g/mL Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM Mg^{2+} and 20 mM glucose) was added to each tube, tubes inverted to mix and incubated for 1 hour at 37 °C with shaking at 225 rpm. One hundred microliters of each transformation mix was spread onto each of two Luria Bertani (LB) agar (10 g/L Bacto-tryptone, 5g/L Bacto-yeast extract, 5g/L NaCl and 15 g/L agar) plates supplemented with 100 μg/mL ampicillin. The remaining transformation mix was pelleted at 16,000 *g* for 2 min, supernatant discarded, cells resuspended in 200 μL SOC medium and 100 μL plated onto each of two LB agar plates supplemented with 100 μg/mL ampicillin. All plates were incubated at 37°C for 48 hours.

2.7 Screening of *E.coli* transformants

Transformants to be used for expression of recombinant BFDV capsid protein were selected by screening for occurrence and orientation of insert in the plasmid vector. Screening was achieved by PCR, restriction digest and Dye-terminator sequencing of the plasmids.

2.7.1 PCR screening of transformants

Isolated colonies were subcultured onto fresh LB agar plates supplemented with 100 μg/mL ampicillin and incubated overnight at 37°C. Between 2 and 10 isolated colonies from each of the 12 plates were picked using a flame sterilized loop, pricked onto a

grided LB agar plate supplemented with 100 μg/mL ampicillin (to be used as a storage library), and then the loop was swabbed in a 0.5 mL microcentrifuge tube containing 50 μL PCR colony buffer [TE pH 8.0, 0.1 % w/v Triton X-100 (Promega, Australia)]. Colonies from the same plate were added to the same tube. Cells, resuspended in PCR colony buffer, were boiled for 5 min and centrifuged for 5 min at 20,800 g to pellet cell debris.

PCR reactions for screening were made to a total volume of 30 μL consisting of 0.7 U Expand High Fidelity DNA polymerase (Roche), 1×polymerisation buffer (composition not supplied, Roche), 2.083 mM $MgCl_2$ (Roche), 300 to 400ng DNA template, 0.083 mM dNTPs, 25-35 ρmol of each primer and made up to 30 μL with Ultra pure water (Fisher Biotec, Australia). Thermal cycle are outlined in Table 2-5. Colonies from the reactions found positive by the pooled PCR screening reactions were further screened individually.

Table 2-5. Thermal cycle protocol used for screening transformants for insert.

Stage	Temperature (°C)	Time (min)	Cycles
1	95	5	1
2	95	0.5	
	50	0.5	30
	46.1	0.5	
	72	1	
3	72	10	1
4	15	HOLD	

2.7.2 Alkaline miniprep technique for purification of recombinant constructs for use in restriction digest screening

Colonies found positive by PCR were grown up to stationary phase in 10 mL 2×YT broth supplemented with 100 μg/mL ampicillin overnight at 37 °C with shaking at 225 rpm. Three millilitres of each cell culture was centrifuged at 10,000 *g* for 30 s to pellet the bacteria. Supernatant was discarded and pellet resuspended in 200 μL resuspension solution [25 mM Tris, 10 mM EDTA pH 8.0, 20 μg/mL Ribonuclease A (Sigma, Australia)]. Two hundred microliters of a solution of 0.2 M NaOH, 1% w/v SDS and distilled water was added and tubes gently inverted 8 times before slowly adding 200 μL of a solution of 1.8M KOA and 11.5% (v/v) glacial acetic acid. Tubes were then gently inverted until white precipitate appeared and then centrifuged for 10 min at 10,000 *g*. Supernatant was transferred to a clean tube and 600 μL of ice cold 100% isopropanol added. Reactions were left to precipitate at room temperature for 15 min. Tubes were then centrifuged for 30 min at 12,000 *g*, supernatant discarded, pellet washed in 200 μL 70% ethanol, DNA pelleted at 12,000 *g* for 5 min, supernatant taken off by aspiration and pellet dried using a Savant speed vac concentrator. Finally, the pellet was resuspended in 50 μL TE buffer (pH 8.0).

2.7.3 Restriction digest screening of pPinPoint™ recombinant construct

Orientation of insert in full length and 3' truncated recombinants was determined by restriction digest. The pPinPoint™ vector has a single *Bam*H I restriction site at nt 406 and the full length insert at nt 584, thus full length recombinants with correctly oriented insert should produce fragments of 598 and 3477 bp when digested with *Bam*H I. Restriction digest of full length recombinants was carried out as follows. Restriction digest reactions were made up to a final volume of 30 μL in 0.65 mL microcentrifuge tubes, consisting of 25 μL plasmid prep, 20 U *Bam*H I (New England Biolabs, Inc),

1×*Bam*H1 buffer (New England Biolabs, Inc), 1×BSA (New England Biolabs, Inc) and made up to 30 μL with distilled water. Reactions were incubated in a 37°C water bath for 1.5 hours.

The 3' truncated recombinants were digested with *Sal* I which cuts the pPinPoint™ vector at nt 52, and with *Sph* I which cuts the insert at nt position 22, thus 3' truncated recombinants with correctly oriented insert should produce fragments of 388 and 3273 bp when digested with *Sal* I and *Sph* I. Restriction digest of 3' truncated recombinants was carried out as follows. Restriction digest reactions were made up to a final volume of 30 μL in 0.65 mL microcentrifuge tubes, consisting of 20 μL plasmid prep, 10 U *Sal* I (New England Biolabs, Inc), 1×*Sal* I buffer (New England Biolabs, Inc), 1×BSA (New England Biolabs, Inc) and made up to 30 μL with distilled water. Reactions were incubated at 37°C for 21 hours. The enzyme was then heat inactivated in a 65°C water bath for 20 min, 5 U *Sph* I added and incubated at 37°C for 1 hour. Results of restriction digest were visualized as outlined under section 2.4.

2.8   Extraction and purification of plasmid for dye-terminator sequencing

The recombinant pPinPoint™ Xa-1 T-vector that contained the correctly sized and oriented insert were purified prior to sequencing using the BioRad Aurum Plasmid Mini Kit, performed exactly as instructed by the manufacturer, using 3 mL of overnight culture grown in 10 mL LB broth supplemented with 100 μg/mL ampicillin. Three millilitres (2×1.5 mL) of this culture was centrifuged for 1 min at 10,000 *g*, supernatant removed by aspiration and pellet resuspended in 250 μL resuspension solution by vortexing. Two hundred and fifty microliters of lysis solution was added and contents of tube mixed by inverting rapidly 8 times. Then, 350 μL of neutralization buffer was added and tubes inverted briskly 8 times and the neutralized lysate centrifuged at 10,000 *g* for 5 min. The supernatant was transferred by pipetting to a plasmid mini column

inserted into a 2 mL cap-less tube, and centrifuged at 10,000 g for 1 min. Filtrate was discarded, 750 μL wash solution added to the mini column and centrifuged for 1 min at 10,000 g. The wash solution was then discarded, column replaced into the tube and centrifuged again at 10,000 g for 1 min to remove residual wash solution. Mini column was then transferred to a 1.5 mL capped microcentrifuge tube, 50 μL elution solution added onto the membrane and allowed to saturate the membrane for 1 min. Finally, the tube was centrifuged at 10,000 g for 1 min to elute the plasmid, and mini column discarded.

2.9   Dye-terminator sequencing

Recombinant plasmid vectors were sequenced by the Dye-terminator sequencing method. This method of sequencing is similar to PCR with the amplification of the DNA; however, in contrast to PCR, dye-terminator sequencing utilizes only one primer. Thus elongation is only terminated when there is incorporation of a fluorescently labelled dideoxynucleotidetriphosphate (ddNTP). Such terminations will be at random, resulting in fragments of varying size labelled at the end. Amplification reactions are electrophoresed, separating the fragments on the basis of molecular weight and allowing the sequence to be determined on the basis of location of each fluorescent ddNTP.

All recombinant plasmids proven to have the insert in correct orientation were sequenced twice, once with the SP6 primer (Table 2-1) and once with the pinseq primer (Table 2-1). Reactions were made to a total volume of 10 μL in 0.2 mL PCR tubes and consisted of 2 μL BigDye terminator mix version 3.1 (Applied Biosystems, Australia, composition not given), 5×sequencing buffer (Applied Biosystems, Australia, composition not given) 3.2 ρmoles of one sequencing primer, 300-400 ng of plasmid

DNA and made up to a final volume of 10 µL with ultra pure water (Fisher Biotec, Australia). Reactions were cycled according to the protocol outlined Table 2-6.

Table 2-6. Thermalcycle protocol used for dye-terminator sequencing of recombinant plasmids.

Stage	Temperature (°C)	Time	Cycles
1	96	5 min	1
2	96	10 s	
	55	5 s	25
	60	4 min	
3	60	10 min	1
4	15	HOLD	

Excess primers, salt and nts were removed by ethanol precipitation of the sequencing products. For each sequencing reaction a 0.5 mL tube containing a solution of 92.6% ethanol, 111.1 mM sodium acetate and 4.6 mM EDTA was prepared. Sequencing products were added, mixed by pipetting and incubated on ice for 20 min. Tubes were microcentrifuged at 20,800 g for 30 min at room temperature, supernatant discarded and pellets rinsed in 125 µL 80% ethanol. Finally, the tubes were centrifuged for 5 min at 20,800 g, supernatant removed and pellet dried under vacuum (SpeedVac Concentrator, Savant). Sequencing reactions were electrophoresed and interpreted by Francis Brigg (State Agricultural Biotechnology Centre sequencing facility, Murdoch University). Briefly; samples were resuspended in 2.25 µL loading dye [1:5 (50 mM EDTA, 50 mg/mL blue dextran): Hi Di Foramide], 0.85 µL of the resuspended sample was loaded into the 5.25% PAGE Plus acrylamide (6 M Urea) gel, and electrophoresed at 2400 Volts, 50 mA, 200 watts, gel temperature 51°C for 13 hours with laser power 40 mW, CCD offset 250, CCD Gain 2 in the sequencer 377XL (Applied Biosystems, Australia)

and gels interpreted with the ABI PRISM 377XL Data Collection version 2.6 and Sequencing Analysis version 3.4.1 (both Applied Biosystems, Australia). Finally, sequences were edited using seqEd v1.0.3 (Applied Biosystems, Australia). All edited sequences were imported into BioManager (www.biomanager.angis.org.au) and analysed using the programmes blastn v2.0 to 2.29 and translate.

2.10 Optimisation of protein expression and cell lysis

Proteins expressed in bacterial systems may be bound up in IBs, termed insoluble protein, thus complicating protein purification. The following experiments were carried out to achieve optimal yield of expressed soluble protein and to determine the best method of lysing cells for achieving the highest overall yield of released protein for purification.

2.10.1 Optimization of induction of full length and C-terminal truncated recombinant protein expression

Cells were transferred using a flame sterilized loop from glycerol stocks to 10 mL of 2×YT broth containing 100 μg/mL ampicillin. Tubes were incubated overnight with shaking (225 rpm) at 37°C. Overnight cultures were then diluted 1:20 with 2×YT containing ampicillin (100 μg/mL) and 2 μM d-Biotin (Sigma, Australia). Cultures were incubated at 37°C with shaking at 225 rpm and induced at optical densities measured at wavelength 600nm (OD_{600nm}) 0.32, 0.62, 0.90 and 1.19 for full length and OD_{600nm} 0.32, 0.59, 0.94 and 1.17 for C-terminal truncated protein with isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma, Australia) at a final concentration of 0.1 mM and culture simultaneously supplemented with d-Biotin to a final concentration of 4 μM. Samples were taken every 30 min for 5.5 hours post induction and additionally

overnight with C-terminal truncated protein. Samples were centrifuged at 20,800 g for 5 min and supernatant discarded.

Pellets were further resuspended in cell lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl and 5% v/v glycerol) at a ratio of 0.1 mL buffer per mL induction culture. Tubes were centrifuged at 20,800 g for 7 min and supernatant discarded. The pellet was resuspended as before and phenylmethylsylphonyl fluoride (PMSF) (Roche, Germany) and lysozyme (Sigma, Australia) added to final concentrations of 1 mM and 1 mg/mL, respectively. Samples were rocked at room temperature for 10 min. Tubes were freeze-thawed using liquid nitrogen and a 42°C water bath 4 times. Triton X-100 was added at a final concentration of 1% (v/v) and tubes vortexed to mix. To separate soluble protein and insoluble IB protein, samples were centrifuged at 20,800 g at 4°C for 20 min. Soluble fraction (supernatant) was then transferred to a clean tube and the insoluble fraction (pellet) was re-suspended in 0.1 mL cell lysis buffer by vortexing. Optimal cell density and length of induction was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblot as described in sections 2.11 and 2.12, respectively.

All cell densities and lengths of induction resulted in production of insoluble protein, however it was found that at OD_{600nm} 0.62 and 2.5 hours of induction, full length expressing *E. coli* JM109 gave a low level of expressed soluble protein in addition to the stronger fraction of insoluble protein expressed. For C-terminal truncated protein expression, soluble protein could not be produced, however a strong insoluble fraction was found at OD_{600nm} 0.94 with 5 hours induction.

2.10.2 Optimization of cell lysis

Three procedures for cell lysis; lysozyme, sonication and freeze-thaw; were tried in order to determine the most efficient method for lysing JM109 *E. coli* JM109. Full length expressing, C-terminal truncated expressing, non-recombinant JM109 and Jembrana Disease Virus capsid protein expressing (termed Jca) cells were grown up overnight as previously described. These cultures were diluted 1:20 with 2×YT broth supplemented with 100mg/mL ampicillin (except for in the case of *non-recombinant E. coli* JM109), incubated for 1.5 hours at 37°C, d-Biotin added (final concentration 2 μM) and cultures induced at OD_{600nm} 0.81 for full length, 0.72 for 3' truncated, 0.53 for Jca and 0.95 for *non-recombinant* JM109 with IPTG (final concentration 100 μM) overnight. Cultures were centrifuged at 2,000 *g* for 7 min at 4°C, supernatant discarded and pellets resuspended in 1 mL cell lysis buffer per 10 mL induced culture by pipetting up and down on ice. PMSF was added to a final concentration of 1mM.

Lysis by lysozyme was performed by adding lysozyme to the resuspended cells at a final concentration of 1 mg/mL. The solution was then rocked for 2 hours at room temperature. Lysis by sonication was performed with sonicator XL2015 (Unimed, Australia) using 50% pulse cycles of 2×2 min at 3.5 microtip. For freeze-thaw lysis, lysozyme was added to a final concentration of 1 mg/mL. Tubes were submerged in liquid nitrogen until solidified and then thawed in a 42°C water bath. Cycle was repeated 10 times. Efficiency of lysis treatment was determined by microscope (Olympus microscope CH-2) examination at 400× magnification. Sonication was found to be the most efficient method for lysing full length and C-terminal truncated protein expressing *E. col* JM109*i*. However, *non-recombinant* JM109 cells and Jca expressing JM109 cells were more efficiently lysed by the freeze-thaw method.

2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was carried out in vertical gels with 12.43% acrylamide (BioRad, Australia). Protein suspensions were made up with 1×loading dye (62.5 mM Tris-HCl, 10% v/v glycerol, 2% w/v sodium dodecyl sulphate, 5% v/v mercaptoethanol, 0.5% w/v bromophenol blue), loaded into the SDS-PAGE gel and electrophoresed for 1 hour at 200 Volts in 1×running buffer [3.47 mM SDS (BDH, Australia), 191.8 mM Glycine (BDH, Australia), 24.8 mM Tris-HCl (Progen Biosciences, Australia)] using the BioRad gel system. The size of separated proteins was determined by comparing with Precision Plus Protein Standards Dual colour (BioRad, Australia). To visualize protein bands, gels were stained with Coomassie Blue (45% methanol, 10% glacial acetic acid, 0.1% w/v Coomassie Brilliant Blue) overnight and excess stain removed by soaking in de-stain (40% methanol, 10% glacial acetic acid, 50% distilled water) overnight. Imaging was performed using a Minolta Dimage Xt digital camera and image contrast and brightness enhanced with Paint Shop Pro 6.

2.12 Western immunoblot

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Australia) using a transblot apparatus (BioRad, Australia) or using a Transblot SD semidry transfer cell (BioRad, Australia). Nitrocellulose membrane, SDS-PAGE gel and Whatman filters were equilibriated for 20 min in western transfer buffer [20% methanol, 25 mM Tris (Progen Biosciences, Australia), 192 mM glycine (BDH, Australia)] prior to transfer. Transfer was carried out overnight at 40V in western transfer buffer when using transblot apparatus and 30 min and 15 Volts with the semi dry apparatus. Membranes were incubated in blocking solution [1×TBS, 0.05% v/v Tween (Sigma, Australia), 5% w/v skim milk powder] overnight at 4°C or for 1 hour at 37°C. Blocking solution was removed, and bound protein reacted with streptavidin-

alkaline phosphatase, chicken-anti-BFDV sera or cockatoo-anti-BFDV as described in sections 2.12.1, 2.12.2 and 2.12.3, respectively. Imaging was performed using a Nikon digital camera and image contrast and brightness were enhanced with Paint Shop Pro 6.

2.12.1 Streptavidin-alkaline phosphatase

The nitrocellulose membrane was incubated in blocking solution containing streptavidin-alkaline phosphatase (Promega, Australia) at a dilution of 1:5000 or 1:2000 with blocking solution for 1 hour at 37°C, washed 2×10 min with 1×TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% v/v Tween 20) and 10 min with 1×TBS (10 mM Tris-HCl, 150 mM NaCl) before adding Western Blue (Promega, Australia)-the substrate for alkaline phosphatase.

2.12.2 Chicken-anti-BFDV sera

Chicken-anti-BFDV sera with an HI titre of 5120 HI units (HIU) was provided by Dr Shane Raidal. The membrane was incubated with chicken-anti-BFDV sera at a dilution of 1:200 in 1×TBST overnight at room temperature and washed 2×10 min in 1×TBST and 10 min with 1×TBS to remove excess antibody. Horseradish peroxidase (HRP) conjugated rabbit-anti-chicken IgG (MP Biomedicals, Inc) was diluted 1:2000 with TBST and incubated with the membrane for 2 hours at room temperature, membrane washed as described previously and bound antibody visualized by adding HRP substrate [0.5 mg per mL HRP colour development reagent (BioRad, Australia), 16.7% v/v Methanol, 83.3% v/v TBS, 0.015% v/v H_2O_2].

2.12.3 Cockatoo-anti-BFDV sera

Cockatoo-anti-BFDV sera with an HI titre of 5120 HIU was provided by Dr Shane Raidal. The membrane was incubated with cockatoo-anti-BFDV sera at a dilution of 1:200 in TBST overnight at room temperature and washed 2×10 min in 1×TBST and 10

min with 1×TBS to remove excess antibody. Further, the membrane was incubated with goat-anti-cockatoo sera (supplied by Dr Shane Raidal) diluted 1:200 in 1×TBST for 2 hours at room temperature and then washed 2×10 min in 1×TBST and 10 min in 1×TBS. HRP conjugated rabbit-anti-goat IgG (MP Biomedicals, Inc) was diluted 1:2000 with 1×TBST and incubated with the membrane for 2 hours at room temperature. Membrane was washed as described previously and bound antibody visualized by adding HRP substrate.

2.13   Purification of full length recombinant BFDV protein from *E. coli* JM109

As mentioned, foreign proteins expressed in bacterial systems commonly become tied up in inclusion bodies thus resulting in an insoluble protein fraction. Optimization experiments resulted in proteins being expressed as both a soluble form and an insoluble form of the expressed full-length recombinant protein. Thus the purpose of the following purification experiments was to gain pure soluble protein and to purify and solubilize IB protein.

2.13.1 Soluble fraction

Soluble fraction was attempted to be purified by both batch capture and column capture methods. In both instances, 500 μL Softlink™ Soft Release Avidin Resin (Promega, Australia) was used per 20 mL soluble fraction obtained. Resin was prepared before use by pre-adsorbing nonreversible binding sites with 2 resin bed volumes of cell lysis buffer containing 5 mM d-Biotin. The buffer/resin mix was left at room temperature for 15 min, buffer removed and resin regenerated by washing in 8 resin-bed volumes of 10% acetic acid, and then in 8 resin bed volumes of 100 mM phosphate buffer (pH 7.0) until the solution reached pH 6.8. Protein was allowed to bind overnight at 4°C on a vertical rotor.

2.13.1.1 Batch capture

All steps were performed at 4°C. The suspension of overnight binding was centrifuged at 500 *g* for 10 min at 4°C to collect resin at the bottom of the tube and supernatant removed by aspiration. The supernatant was kept to determine the proportion of unbound protein by SDS-PAGE and western immunoblot. Resin was washed in 1 mL ice cold cell lysis buffer per 25 μL of resin by inverting 10 times and then centrifuging the sample at 500 *g* for 10 min at 4°C. The supernatant was aspirated and the wash process repeated 2 more times in 0.5 mL ice cold cell lysis buffer per 25μL resin. All 3 washes were kept to determine, using SDS-PAGE, whether the wash steps removed the recombinant protein or just contaminants. One millilitre elution solution (cell lysis buffer supplemented with 5 mM d-Biotin) was added per 250 μL resin and eluted at 4°C for 1 hour on a vertical rotor (elution 1). The elution was repeated a second time for approximately 5 hours (elution 2) and then finally overnight (elution 3). All 3 elutions were stored as they were expected to contain pure recombinant protein to be used in vaccination and were analysed by SDS-PAGE to determine such.

2.13.1.2 Column capture

All steps were performed at 4°C. The suspension from overnight binding was loaded into a sterile 0.8×4 cm Poly-prep chromatography column (BioRad, Australia) and allowed to flow-through. Flow-through was passed through the column 3 more times. The flow-through was stored and analysed by SDS-PAGE and western immunoblot to determine the amount of unbound recombinant protein. Resin was washed by adding 1 mL ice cold cell lysis buffer per 25 μL resin and inverting 10 times to completely disrupt the resin bead bed. The buffer was allowed to flow through, 0.5 mL fresh ice cold buffer added per 25 μL resin, and column left to allow for flow-through. Resin was washed a third time as for the second time. All washes were analysed for presence of

recombinant protein by SDS-PAGE. Elution solution was added at a ratio of 1 mL elution solution per 25 μL resin, allowed resin to settle for 1 hour and elutant collected in 0.5 mL fractions (elution 1 and elution 2). One millilitre elution solution was added and collected immediately in 0.5 mL fractions (elution 3 and elution 4). Finally, the resin was left in 1 mL elution solution per 25 μL resin overnight and then allowed to flow-through (elution O/N). All elutions were stored and analysed by SDS-PAGE.

Both the column and the batch method proved useless for purification of soluble protein as the biotinylated protein did not bind to the resin.

2.13.2 Insoluble fraction

Two methods were found useful for the purification and solubilization of IB protein; B-PER and Urea/DTT method. The latter however, was considered the most practical and time efficient.

2.13.2.1 B-PER

All steps were performed on ice. Pellet of 400 mL lysed culture was resuspended in 5 mL of cell lysis buffer and then added 20 mL of 1:10 dilution of B-PER (wash 1). Tube was centrifuged (Beckman centrifuge J-30 I) at 16,000 g for 30 min in a 4°C pre cooled rotor (Beckman rotor JA 25-50). Supernatant was taken off and pellet resuspended in 1:20 diluted B-PER (wash 2) and centrifuged as before, supernatant taken off and pellet resuspended in 1 mL of cell lysis buffer, then 9 mL buffer added (wash 3) and centrifuged again. The pellet was finally resuspended in 1 mL 1×PBS. All washes and the final product were stored and analysed by SDS-PAGE.

2.13.2.2 Urea/DTT solubilisation

The insoluble fraction used in this procedure was obtained as for the B-PER procedure, with the exception of French press being used for cell lysis in place of sonication, due to

sonicator being out of order. Three millilitres of wash buffer (10 mM Tris-HCl, 5 mM EDTA, 1% v/v Triton X-100) was added per 40 ml pelleted lysate and resuspended by vortexing, shaking, pipetting and using the vertical rotor at 4°C (wash 1). The suspension was centrifuged at 10,000 g for 15 min at 4°C (pre cooled Beckman rotor JA 25-50), and supernatant taken off. Three millilitres of wash buffer and 10 µg deoxyribonuclease 1 (Sigma, Australia) was added and the pellet resuspended as before (wash 2). Tubes were centrifuged again, supernatant taken off and pellet resuspended in 3 mL wash buffer (wash 3), centrifuged (10,000 g, 15 min) and supernatant taken off. Finally, the pellet was resuspended in 10 mL solubilization buffer [2 M Tris-HCl, 2M Urea, 0.4mM dithiothreitol (Progen, Australia), 1 mM PMSF, pH 12] per 40 mL pelleted lysate. All wash fractions and the final product were analysed by SDS-PAGE.

2.14 Inoculation of sheep with recombinant protein

Two sheep were inoculated with recombinant protein. Inoculum was prepared immediately before injection by homogenizing Urea/DTT solubilised full-length protein (prepared as described above) with Freund's incomplete adjuvant at a ratio of 1:1 using 2 sterile glass syringes connected to each other with a sterile steel tube. Both animals were bled from the jugular vein prior to injection. Prepared inoculum was injected subcutaneously into the left gluteal area and the dorsal neck with 2 mL vaccine in each site for animal number 761 and 1.5 mL for animal number 5.

Twenty one days post injection; both animals were bled and then boosted with 2.5 mL inoculum subcutaneously in both the left rump and the dorsal neck. Both animals were bled 27 days post boosting and then boosted again at the same sites as before with 3 mL for sheep 761 and 2.5 mL for sheep 5. Both animals were bled a last time at 16 days after the second boost. All bloods were collected into sterile plain glass containers and

allowed to clot. Serum was collected and heat inactivated at 57°C for 30 min and then stored at −20°C. Antibody responses were detected by western immunoblot and IHC as described in sections 2.16.1 and 2.16.2, respectively.

2.15 Vaccination of psittacine birds

Inoculum was prepared as described for sheep and 0.1-0.2 mL was injected into the pectoral muscle mass of 12 psittacine birds. Of these birds, 1 was boosted twice, 3 boosted once and the remaining 9 only received primary vaccination. Blood samples were collected onto filter paper (Whatman No. 3) before injection, 11 or 14 days post injection, 32 or 46 days post the first boost and 14 days post the second boost. Antibody response was detected as per Riddoch and Raidal (1996) and outlined in section 2.16.3.

2.16 Detection of antibody-response to recombinant BFDV protein by western immunoblot, IHC and HI

Detection of antibody response in sheep and birds was attempted by HI as described by Riddoch *et al.* (1996), however this did not give any results for the sheep samples because the sheep sera caused low levels of auto-agglutination interfering with the principles of the assay. Due to this complication, western immunoblot and IHC was employed for analysing sheep sera.

2.16.1 Western immunoblot detection of antibodies to full length recombinant protein in sheep sera

One hundred and fifty microliters of urea/DTT solubilised IB protein was electrophoresed as described under section 2.9 and transferred to a nitrocellulose membrane as described under section 2.10. Sera collected in section 2.13 was diluted 1:200 with 1×TBST and incubated with the membrane over nigh at room temperature, washed 2×5 min in 1×TBST and 10 min in 1×TBS and then incubated with HRP

conjugated rabbit-anti-sheep IgG diluted 1:2000 with 1×TBST. Membrane was washed as before and HRP colour development was carried out as described under section 2.12.2. Imaging was performed using a Nikon digital camera. Image contrast and brightness were enhanced with Paint Shop Pro 6.

2.16.2 IHC detection of antibodies to full length recombinant protein

Sections of formalin-fixed and paraffin embedded skin tissue from a chronically infected cockatoo were cut to a thickness of 5μm using a Leica RM 2135 microtome and placed onto a glass slide. The sample was then dewaxed 3 times in xylene for 3 minutes and further rehydrated using decreasing ethanol concentrations [100%, 95% and 70% (v/v)] for 3 min in each solution. Sections were then washed for 3 min in Tris buffer twice. Endogenous peroxides were removed by submerging the slides in a solution of 0.3% (v/v) hydrogen peroxide in methanol for 5 min and then washed 3×3 min in Tris buffer. Slides were further incubated in blocking solution (DAKO® Protein Block) for 30 min at room temperature in a humidified environment. Sheep sera diluted 1:50 in antibody diluent (DAKO®) was incubated with the slides at room temperature and unbound antibody was then removed by washing 3×3 min in Tris buffer before incubating with biotin conjugated rabbit-anti-sheep diluted 1:1000 in a humidified environment at room temperature for 15 min. The slide was washed as before and then incubated with HRP-conjugated streptavidin for 10 min at room temperature in a humidified environment before washing as previously described and antigen-antibody complexes visualised with HRP colour development as described in section 2.12.2. Imaging was performed using an Olympus BX 13 microscope and digital camera accessory. Image contrast and brightness were enhanced with Shop Pro 6.

2.16.3 HI detection of antibodies to full length recombinant protein in psittacine bird sera

A hole punch was used to clip out a piece of filter paper with blood, which was dropped into 100 μL 5% acid washed kaolin and left to elute for 1 hour at room temperature or overnight at 4°C. The sample was briefly centrifuged to pellet the kaolin and paper. Fifty microliters of supernatant was transferred to a new tube and mixed with 50 μL 10% galah erythrocytes and allowed to haemadsorb overnight at 4°C. Further, 50 μL PBS was added to every well of a microtiter plate and then 50 μL sample added to the first lane and serially diluted 1:2 across the lane. The microtiter plate was then incubated at 37°C for 60 min and 50 μL erythrocytes added to each well and incubated for 60 min at 37°C. Imaging was performed using a Nikon digital camera and image contrast and brightness enhanced with Paint Shop Pro 6.

3   Results

3.1   PCR amplification of full length, 3' truncated and 5' truncated BFDV ORF C1.

PCR amplification of full length, 3' truncated and 5' truncated BFDV ORF C1 (Figure 3-1) was performed using optimized conditions, as described in Chapter 2, section 2.3. Three negative control reactions, 2 using DNA extract from BFDV negative psittacine birds and 1 water control was used. Amplification resulted in the desired 744, 330 and 649 bp fragments for full length (lane 2), 3' truncated (lane 7) and 5' truncated (lane 12) amplification, respectively. None of the control reactions resulted in amplification.
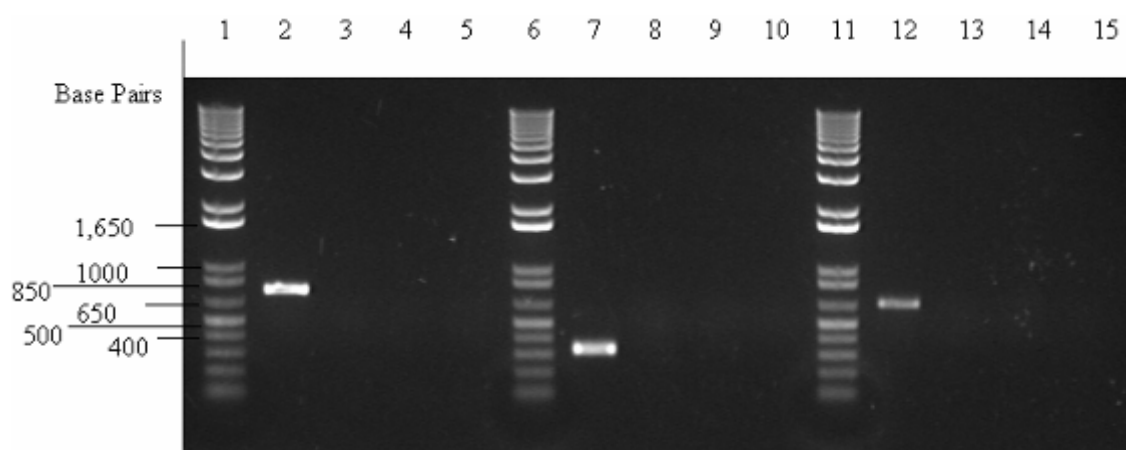
Figure 3-1. PCR amplification of BFDV full length, 3' truncated and 5' truncated ORF C1. PCR products were electrophoresed in a 1.0% agarose gel at 90V for 30 min. Lane 1: Invitrogen 1 Kb plus DNA ladder. Lane 2: Full length amplification of ORF C1. Lane 3: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 4: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 5: Water control. Lane 6: Invitrogen 1 Kb plus DNA ladder. Lane 7: 3' truncated amplification of ORF C1. Lane 8: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 9: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 10: Water control. Lane 11: Invitrogen 1 Kb plus DNA ladder. Lane 12: 5' truncated amplification of ORF C1. Lane 13: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 14: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 15: Water control.

3.2   Screening of Transformed *E. coli* JM109

3.2.1   PCR and restriction digest screening of transformants

Full length and 5' truncated transformants were screened by PCR using the primers Reverse 1 and Pinseq (Table 2-1). Thus full-length and 5' truncated colonies carrying the insert in the correct orientation were expected to give PCR product of 839 and 744 bp, respectively. The 3' truncated recombinants were screened by PCR with the primers Reverse 2 and Pinseq (Table 2-1) and expected to result in a fragment of 425 bp.

PCR screening detected both full length and 3' truncated recombinants. However, repeated PCR screening of 5' truncated colonies did not detect any positives. Full length and 3' truncated colonies found positive by PCR screening were further confirmed by restriction digest to have correctly ligated insert. These colonies were further sequenced by dye-terminator sequencing.

3.3   Dye-terminator Sequencing

All colonies found positive by both PCR and restriction digest were further sequenced with *Dye*-terminator sequencing to confirm whether the recombinants had correctly inserted amplicon in the correct reading frame. Both full length and 3' truncated recombinants carrying the insert in the correct orientation and reading frame were found (Figure 3-2 and Figure 3-3, respectively). These colonies were then inoculated in LB broth supplemented with ampicillin overnight to make glycerol stocks and to be used in protein expression experiments.

```
tca gct ggg atc cgg tac cga ttC ATG TGG GGC ACC TCT AAC TGC GCA TGC GCT
ser ala gly ile arg tyr arg phe met trp gly thr ser asn cys ala cys ala

GTA TGT TCA ATT CCG TCA GTT TGC CCC TAA CAA TCC CAG CAC TTA Aat cag atc tcc
val cys ser ile pro ser val cys pro OCH gln ser gln his leu asn gln ile leu
```

Figure 3-2. Results of dye-terminator sequencing of a full length recombinant BFDV ORF C1. Sequence of nt and a.a. are written 5' to 3'. In red is a portion of the vector sequence flanking the insert, in black are the 5' and 3' ends of insert.

```
cgg tac cga ttC ATG TGG GGC ACC TCT AAC TGC GCA TGC GCT ACA TTT CAG ATT
arg tyr arg phe met trp gly thr ser asn cys ala cys ala thr phe gln ile

ATT TTG AAG ATT ACC GAA TTA AGT TAG CTA AAA TGG AAA TGA GGC CCA atg aga tct
ile leu lys ile thr glu leu ser AMB leu lys trp lys OPA gly pro ser arg ser
```

Figure 3-3. Results of dye-terminator sequencing of a 3' truncated recombinant BFDV ORF C1. Sequence of nt and a.a. are written 5' to 3'. In red is a portion of the vector sequence flanking the insert, in black are the 5' and 3' ends of insert.

3.4 Optimisation of induction of full length and C-terminal truncated recombinant protein expression

3.4.1 Full length protein expression

Recombinant *E. coli* JM109 cells for expressing full length BFDV capsid protein were grown to stationary phase, diluted 1:20 in 2×YT broth and grown to OD_{600nm} 0.32, 0.62, 0.90 and 1.19, and induced with IPTG for 5.5 or 6.0 hrs with samples taken every 30 mins after induction. Cultures were then pelleted by centrifugation and cells lysed by freeze-thaw before separating soluble and insoluble protein fractions. The fractions were further electrophoresed in SDS-PAGE gels before transferring to nitrocellulose membranes and recombinant protein visualized by incubating the membrane with alkaline phosphatase conjugated streptavidin.

A full set of results from this optimisation experiment can be seen in Appendix A. Virtually all cell densities and lengths of induction, including non-induced cell culture (Appendix A, Figure A.1, lane 2), produced insoluble protein. An OD_{600nm} of 0.62 (Figure 3-4, below) with 1.5 and 2.5 hrs of induction (lanes 8 and 12, respectively) clearly gave the highest yield of insoluble protein. In addition, this cell density with the latter induction time gave the highest soluble protein yield (lane 11).

Figure 3-4. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 0.62. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively.

3.4.2 *C-terminal truncated protein expression*

Recombinant *E. coli* JM109 cells for expressing C-terminal truncated BFDV capsid protein were grown to stationary phase, diluted 1:20 in 2×YT broth and grown to OD_{600nm} 0.316, 0.591, 0.940 and 1.174, and induced with IPTG for 5.5 hrs with samples taken every 30 mins after induction and then overnight. Cultures were then pelleted by centrifugation and cells lysed by freeze-thaw before dividing into soluble and insoluble protein fractions. The fractions were further electrophoresed in SDS-PAGE gels before

transferring to nitrocellulose membranes and recombinant protein visualized by incubating the membrane with alkaline phosphatase conjugated streptavidin.

A complete set of results for this optimisation experiment can be viewed in Appendix B. Virtually all cell densities and lengths of induction, including non-induced cell culture (Appendix B, Figure B.1, lane 2) produced insoluble protein. An OD_{600nm} of 0.940 (Figure 3-5, below) gave the highest yield of insoluble protein, with 3.5, 4.0, 4.5 and 5.0 hrs (lanes 2, 4, 6 and 8) of induction giving approximately the same yield. None of the cell densities or lengths of induction resulted in expression of soluble protein.
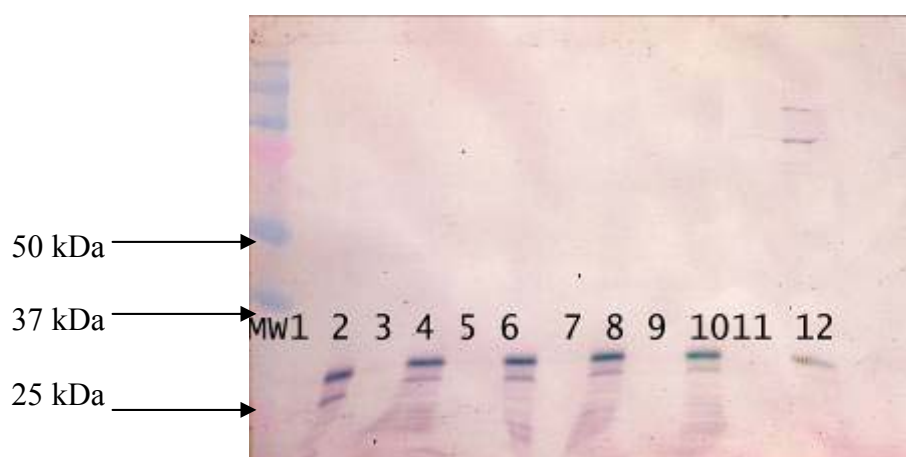
Figure 3-5. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.940. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

3.5 Purification of soluble full length recombinant BFDV protein

3.5.1 Full length soluble protein batch capture

Figure 3-6 illustrates the protein content of fractions taken during purification using the batch capture method. Lane 1 shows protein expression before induction and indicates, as was found in the optimisation of expression experiments, some leaky expression of recombinant protein. Crude lysate of induced cell culture (lane 2) featured a similar banding pattern to lane 1, however weaker. Electrophoresis of non-purified soluble fraction (lane 3) and the protein fraction that did not bind to the Softlink™ Soft Release Avidin Resin (lane 4) gave results almost identical to that of non-induced culture. The first wash (lane 5) removed some *E. coli* proteins, however a significant amount of the recombinant protein was also removed. The 2 subsequent washes (lanes 6 and 7) removed little *E. coli* proteins. Finally, protein elution (lanes 8, 9 and 10) released very little of the recombinant protein, and revealed the insufficiencies of this method for protein purification.

Figure 3-6. SDS-PAGE of full length soluble protein produced with cell culture OD_{600nm} 0.62 and 2.5 hrs of induction. Protein was purified using the batch capture method and the following fractions were separated by SDS-PAGE and visualized with Coomassie blue stain. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Sample of non-induced culture. Lane 2: Non-purified sample after 2.5 hrs of induction. Lane 3: Non purified soluble fraction. Lane 4: Fraction that did not bind to Softlink™ Soft Release Avidin Resin. Lane 5: Wash 1. Lane 6: Wash 2. Lane 7: Wash 3. Lane 8: One hr elution. Lane 9: 5 hr elution. Lane 10: Overnight elution.

3.5.2 *Full length soluble protein column capture*

Figure 3-7 displays the protein content of samples taken during protein purification using the column capture method. The fraction of protein that did not bind to the avidin resin, seen in lane 1, was dense with proteins of various sizes including the size of the desired protein. The first wash (lane 2) removed some *E. coli* proteins and some of the recombinant protein. However, in both instances less protein was removed in this wash than in the equivalent in the batch capture method. The 2 subsequent washes (lanes 3 and 4) did not remove any protein. All 5 elutions (lanes 5, 6, 7, 8 and 9) released a small amount of correctly sized protein, however, as for the batch purification method; the elutions were not pure.
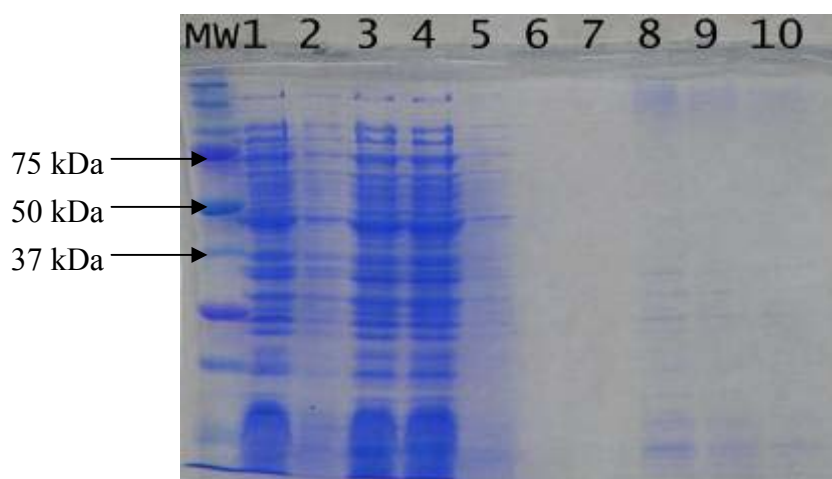
Figure 3-7. SDS-PAGE of full length soluble protein produced with cell culture OD_{600nm} 0.62 and 2.5 hrs of induction. Protein was attempted purified using the column capture method and the following fractions were separated by SDS-PAGE and visualized with Coomassie blue stain. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Fraction that did not bind to Softlink™ Soft Release Avidin Resin. Lane 2: Wash 1. Lane 3: Wash 2. Lane 4: Wash 3. Lane 5: First elution. Lane 6: Second elution. Lane 7: Third elution. Lane 8: Fourth elution. Lane 9: Overnight elution.

3.6 Purification of insoluble full length recombinant BFDV protein

Insoluble full length IB protein was purified and solubilised using the Urea/DTT (Figure 3-8, lanes 1-5) and the B-PER method (Figure 3-8, lanes 6-10). In both methods, the resuspended pellet (Urea/DTT: lane 1 and B-PER: lane 6) was densely populated with proteins of varying molecular weight. Washing the pellet (lanes 2-4 and 7-9) removed some E. coli proteins and some protein at the molecular weight of the recombinant protein. The final products of both methods (lanes 5 and 10) were found to be highly impure.
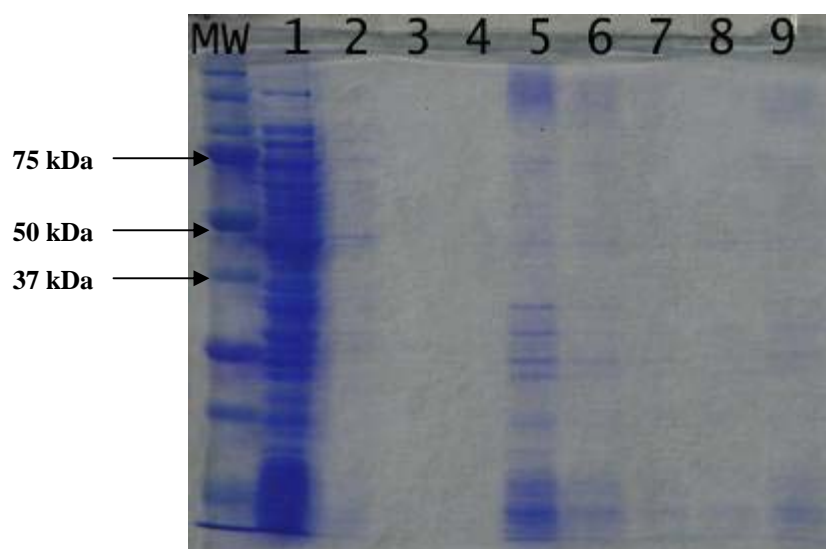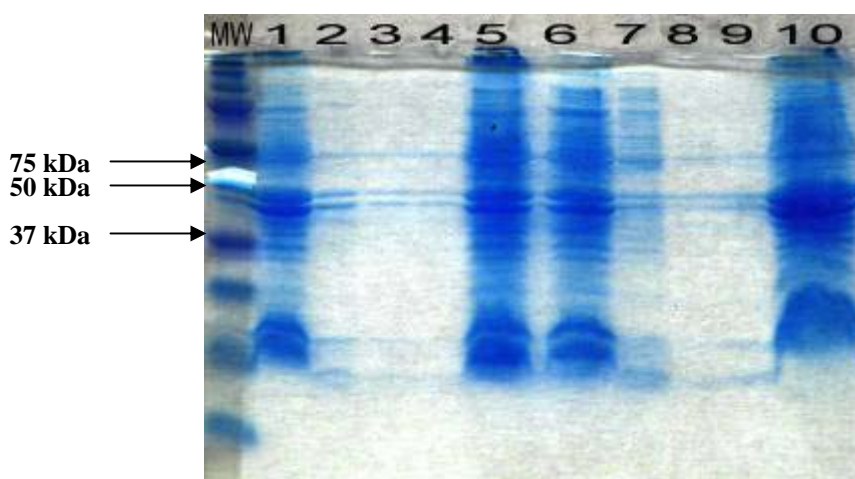
Figure 3-8. SDS-PAGE of full length inclusion-body protein produced with cell culture OD_{600nm} 0.62 and 2.5 hrs of induction. Protein was attempted purified and solubilised using the Urea/DTT (lanes 1-5) and B-PER methods (lanes 6-10). The fractions were visualized by SDS-PAGE and Coomassie blue staining. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Sample of resuspended pellet for purification with the Urea/DTT method. Lane 2: First wash. Lane 3: Second wash. Lane 4: Third wash. Lane 5: Final product of inclusion-body protein purification and solubilization with the Urea/DTT method, Lane 6: Sample of resuspended pellet for purification with the B-PER method. Lane 7: First wash. Lane 8: Second wash. Lane 9: Third wash. Lane 10: Final product of IB protein purification and solubilization with the B-PER method.

3.7 Detection of full length recombinant protein with anti-BFDV-antibodies

To determine whether the recombinant protein could react with antibodies raised specifically against BFDV, the recombinant protein was electrophoresed in an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with sera from a chicken inoculated with purified virus, non-inoculated chicken sera and sera from a naturally immune cockatoo. To visualize formed antigen-antibody complex, the membrane was further incubated with HRP conjugated anti-chicken-antibody or with goat-anti-cockatoo and then with HRP conjugated anti-goat-antibody. Alkaline phosphatase conjugated streptavidin was used as a positive control, to clearly distinguish antibody binding to the recombinant protein from antibody binding to other antigens, e.g. *E. coli* proteins. Results can be seen in Figure 3-9.
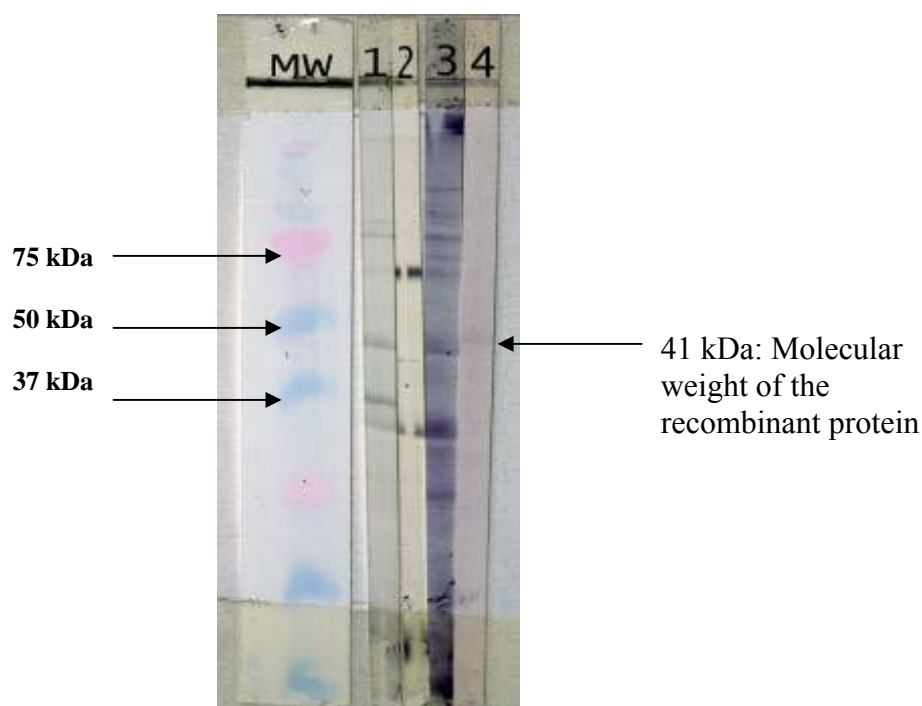
Figure 3-9. Western immunoblot of full length recombinant protein with the following antibodies. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Chicken-anti-BFDV (1:200 dilution). Lane 2: Negative control; non challenged chicken sera (1:200 dilution). Lane 3: Sera from an immune cockatoo (1:200 dilution). Lane 4: Positive control; alkaline phosphatase conjugated streptavidin (1:2000 dilution).

Positive controls using streptavidin (lane 4) consistently detected the biotinylated recombinant protein located between 37 and 50 kDa. Both the BFDV challenged chicken sera (lane 1) and the non-challenged chicken sera (lane 2) resulted in several bands. However, the challenged chicken sera gave a band at the same molecular weight as the positive control (41 kDa), whereas the non-challenged chicken sera did not. Sera from an immune cockatoo also gave multiple bands, including one at desired molecular weight.

3.8   Detection of antibody-response to recombinant BFDV protein by western immunoblot, IHC and HI

Moderately high titres of non-specific autoagglutination were present in the pre and post inoculation sheep sera which interfered with the principles of the HI assay. Therefore western immunoblot and IHC was used to detect antibody response in sheep.

3.8.1   Western immunoblot detection of antibodies to full length recombinant protein in sheep sera

To determine whether the inoculated sheep had raised antibodies against the inoculum, Urea/DTT solubilised recombinant protein was electrophoresed in an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with sera from both sheep pre inoculation, post inoculation and post boosting. Results can be seen in Figure 3-10.
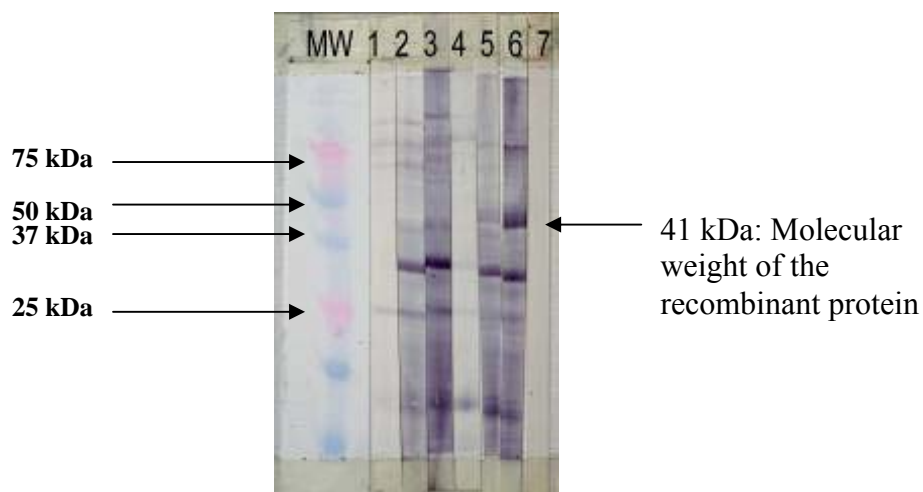
Figure 3-10. Western immunoblot of full length recombinant protein with sera from sheep pre inoculation, post inoculation and post boosting. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Sheep 5 pre inoculation (1:200 dilution). Lane 2: Sheep 5 post inoculation (1:200 dilution). Lane 3: Sheep 5 post boosting (1:200 dilution). Lane 4: Sheep 761 pre inoculation (1:200 dilution). Lane 5: Sheep 761 post inoculation (1:200 dilution). Lane 6: Sheep 761 post boosting (1:200 dilution). Lane 7: Positive control; alkaline phosphatase conjugated streptavidin (1:2000 dilution).

None of the 2 sheep displayed antibody binding at the molecular weight of the recombinant protein (41 kDa) pre inoculation (Figure 3-10, lane 1 and 4). However, after inoculation (lane 2 and 5) both sheep produced antibodies that bound to the recombinant protein. This binding was increased after boosting (lanes 3 and 6).

3.8.2 IHC for detection of antibodies to native BDFV raised in sheep inoculated with recombinant BFDV capsid protein.

IHC was employed to determine whether inoculation of sheep with recombinant BFDV capsid protein would cause the sheep to raise antibodies that would recognise native BFDV. The results of IHC may be seen in Figure 3-11. Sera from sheep post (Figure 3-11A) and pre (Figure 3-11B) inoculation and a skin section from a chronically infected cockatoo were used for this procedure. As can be seen in Figure 3-11A, sera from inoculated sheep recognised BFDV inclusion bodies in the skin section (black arrow), whereas sera harvested from sheep pre inoculation did not react with the viral inclusions (Figure 3-11B).
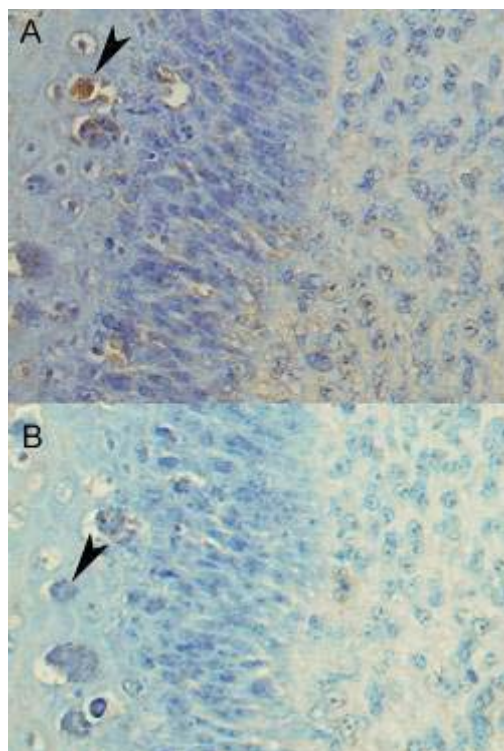
Figure 3-11. IHC using a section of skin from a chronically BFDV infected cockatoo. A) Skin section incubated with sera from inoculated sheep, arrow indicates location of a viral IB where an antigen-antibody complex formed has been visualised by binding of secondary antibody. B) Negative control. Skin section from chronically infected cockatoo incubated with sera from sheep pre inoculation. Arrow points to an example of a BFDV inclusion that was not recognised by the sera.

3.8.3   *HI detection of antibodies to BFDV raised in psittacine birds vaccinated with recombinant BFDV capsid protein.*

Twelve psittacine birds were vaccinated with the solubilised full length IB protein. Antibody response was determined by HI assay. An example of HI assay results may be viewed in Figure 3-12 and a full set of results can be seen in Table 3-1. Three birds (red tailed black cockatoo samueli, eastern long billed corella and galah-corella cross) displayed an increase in anti-BFDV-antibody titre. The red tailed black cockatoo had no antibody pre vaccination and its antibody titre has increased to 80 HI units (HIU) 11 days after vaccination. Boosting the animal resulted in an antibody titre of 160 HIU 32 days later. Similarly, the eastern long billed corella also showed no evidence of anti-BFDV-antibody before vaccination and the animal's antibody titre was at 20 HIU 11

days after vaccination. Unfortunately this bird was not available for further vaccine work, thus monitoring further increase in HIU was not possible. The third bird, a galah-corella cross, had an antibody status of 80 HIU before vaccination. Eleven days post vaccination; this bird had an increase in antibody titre to 160 HIU. Boosting this animal did not result in a further increase in antibody.
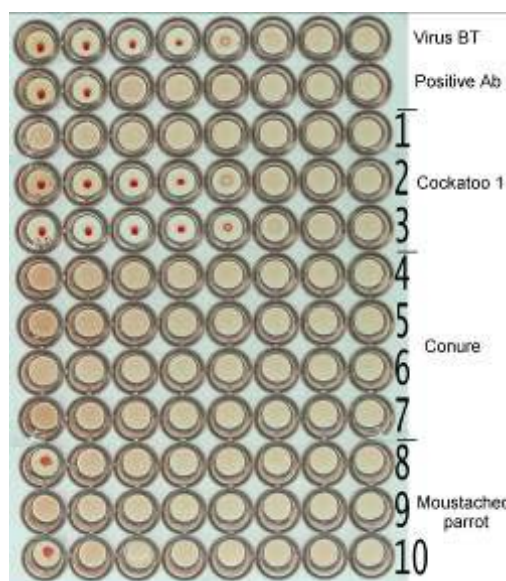
Figure 3-12. Example of microtitre plate showing results of HI assay of three birds vaccinated with solubilised full length recombinant IB protein. Rows 1, 2 and 3: Sera from the red tailed black cockatoo pre vaccination, 11 days post vaccination and 32 days post boosting, respectively. Rows 4, 5, 6 and 7: Sera from a conure pre vaccination, 11 days post vaccination, 32 days post vaccination and 46 days post vaccination, respectively. Rows 8, 9 and 10: Sera from vaccinated moustached parrot 1 pre vaccination, 11 days post vaccination and 14 days post boosting, respectively. Column 1 is a 1:20 dilution of sera. Subsequent columns are serially diluted 1:2 of the column to its left.

Table 3-1. Results of HI screening of experimentally vaccinated birds pre vaccination, post vaccination and post boosting. Blood was collected on the day of vaccination and a number of days (as labelled) after vaccinations and boosting. n/s: no sample taken that day. N/A: bird no longer available for experiment. "Auto-agg": the HI result could not be considered positive or negative due to sera causing auto-agglutination. Days: refers to number of days post the last injection.

Species	Pre inoculation	Post inoculation						
	HI result	Days	HI result	Days	Bosted Y/N	HI result	Days	HI result
Redtailed black cockatoo Samueli	0	11	80	32	y	160	N/A	
Moustached parrot 1	0	11	0	32	y	n/s	14	0
Muostached parrot 2	Auto-agg	11	0	32	y	n/s	14	Auto-agg
Conure	0	11	0	32	n	0	46	0
Eastern long billed correla 1	0	11	20	N/A				
Derbyan	Auto-agg	11	Auto-agg	N/A				
Galah-Corella cross	80	11	160	32	y	160	N/A	
Sulphur crested cockatoo/Corella	80	11	40	46	n	80	N/A	
Eclectus parrot male	0	11	0	N/A				
Eclectus parrot female	0	11	Auto-agg	N/A				
Galah/Major Mitchell	160	14	160	N/A				
Eastern Long Billed Corella 2	160	14	Auto-agg	N/A				

4   Discussion

During this research project a biotinylated recombinant BFDV capsid protein, recognized by anti-BFDV-antibodies raised in chickens and cockatoos, was successfully expressed in a bacterial system. Two sheep and several psittacine birds were injected with the recombinant protein and their antibody response monitored by western immunoblot, IHC and HI. It was found that both sheep and birds inoculated with the recombinant protein induced the production of antibodies that specifically recognised native BFDV. These results show that the recombinant protein is antigenic and that native epitopes of the BFDV have been conserved. This recombinant protein has valuable potential future applications in immunisation of parrots, lorikeets and cockatoos and as antigen in ELISA and HI assays without the requirement of native virus preparation and as a tool for the production of monoclonal antibodies. In addition, this experiment has resulted in a large stock of sheep-anti-BFDV sera that can be used for consistent IHC, HI assays and western immunoblot. The capsid protein was the chosen element for the development of this vaccine as no other structural proteins have been found in association with BFDV.

4.1   PCR amplification of BFDV ORF C1 and screening of recombinant *E. coli* JM109.

PCR reactions were successfully optimised for amplification of full length, 3' truncated and 5' truncated BFDV ORF C1. Amplicons were further ligated into the pPinPoint™ Xa-1 T-vector and the recombinant plasmid successfully transformed into *E. coli* JM109. The full length and 3' truncated amplicons were drmined by PCR and restriction digest to be successfully ligated into the pPinPoint™ Xa-1 T-vector in the

correct orientation. Further screening by dye-terminator sequencing confirmed orientation of insert and desired reading frame.

Recombinant plasmids with correctly inserted 5' truncated amplicon could not be detected during screening experiments. This may have been due to a number of factors. An insert must have been present in the vector and the vector must have been successfully transformed into the cell because these transformants were able to form colonies in the presence of ampicillin. Ampicillin resistance of *E. coli* JM109 is conferred only by the introduced vector. The linear pPinPoint™ Xa-1 T-vector has deoxythymidine overhangs at the 3' ends and can therefore not recircularise in the absence of insert. Linear plasmids are unable transform into bacteria. Ergo the inability to detect 5' truncated recombinants must have been due to incorrectly inserted amplicon and not absence of insert. Only a limited number of 5' truncated recombinants were observed on the agar plates, none of these were found to contain correctly orientated insert. Because of the vector's deoxythymidine overhangs at the 3' ends and the deoxyadenine overhangs of the amplicon, the amplicon can only insert in 1 of 2 possible orientations. In light of this it might seem logical to assume that as much as 50% of the transformants could contain correctly inserted amplicon. Then why were no 5' truncated recombinants detected during screening? The *E. coli* used in this experiment were determined to have leaky expression (see section 3.4 optimisation of protein expression) despite having the *lacI^q* allele which causes overexpression of the lac repressor and should therefore not be subject to leaky expression (Sambrook and Russel, 2001). Cells containing correctly inserted 5' truncated insert may therefore have been expressing the protein while growing on the transformation plates and the expressed protein might impose growth limitations on the cell, resulting in minute colonies not detectable during viewing of plates. These colonies were therefore not picked and screened. This problem may be solved by trying a number of different *E. coli* strains for initial transformation.

This would allow for identification of clones with the right plasmid construct which could then be purified and then introduced into an appropriate strain of bacteria for protein expression. However, if the problem does not lie in growth inhibiting properties of basal recombinant protein expression, subcloning might be required for increasing occurrence of correctly oriented insert. Due to time constraints and the successful cloning of 2 amplification products, the full length and the 3' truncated, it was decided to focus on these, as protein expression of these would include all epitopes of the capsid protein that could be antigenic.

4.2   Optimisation of full length and C-terminal truncated protein expression

Induction of protein expression in *E. coli* JM109 under the control of IPTG was successful for both full length and C-terminal truncated recombinant protein. Rate of cell growth is believed to be an important influence on expression of foreign proteins in bacteria and other than optimal conditions can overload the bacterial protein synthetic apparatus, promoting IB formation. Therefore the density to which cells are grown before induction and after induction is important (reviewed in Sambrook and Russel (2001)). Because of this, optimisation of recombinant full length and C-terminal truncated protein expression was performed in order to obtain soluble protein that could be purified using the Promega PinPoint™ Xa protein purification system and to obtain the highest possible yield of recombinant protein. The length and time of induction was not found to be critical for the expression of recombinant protein in general, however did have an effect on yield of soluble recombinant protein. Optimal protein yield was found at OD_{600nm} 0.62 with 1.5 and 2.5 hours of induction for full length expressing cells and an OD_{600nm} of 0.940 with induction time between 3.5 and 5.0 hours for C-terminal truncated protein expressing cells. Full length soluble protein was obtained under specific conditions of cell density and length of induction, indicating the

importance of cell density and length of induction on expression of soluble protein. Soluble C-terminal truncated recombinant protein could not be obtained under the conditions examined. Trying a broader range of cell densities could possibly overcome this problem. Both full length and C-terminal truncated forms could be expressed in an insoluble form as IBs.

4.3   Purification of recombinant protein

The most immunogenic epitopes of the BFDV capsid protein have not been determined. However, the C-terminal truncated protein was designed on the basis of prediction of a major antigenic epitope, as seen in section 2.3, and could possibly result in a recombinant BFDV capsid protein more antigenic than the full length protein. Initial assessment of the antigenicity of the C-terminal truncated protein compared to the full length protein can in the future be performed using the sheep-anti-recombinant BFDV protein yielded in this project. However the aims of this project were not to characterise antigenic epitopes in the BFDV capsid protein and the recombinant full length protein would cover the complete set of epitopes. This, in addition to time consuming optimisation of full length protein purification, caused C-terminal truncated protein purification attempts to not be pursued.

4.3.1   *Full length soluble protein*

Expressed soluble full length protein was purified from crude lysate of recombinant expressing cells using the Promega PinPoint™ Xa purification system. Both batch capture and column capture were attempted in order to increase the binding of biotinylated recombinant protein to resin. The PinPoint™ Xa system uses Softlink™ Soft Release Avidin Resin for purification. This resin consists of resin beads with attached avidin molecules. Such avidin molecules are intended to bind to biotin - the

fusion tag used in this expression system, allowing for *E. coli* proteins and cellular debris to be removed by washing (Promega, 2001).

Separation of the different protein fractions collected during batch capture by SDS-PAGE revealed that the non-purified fraction and the non-bound fraction had an almost identical protein content including that of the recombinant protein. This indicates that the resin had low capacity to bind the biotinylated recombinant protein, despite the high affinity ($K_d \sim 10^{-15}$ M) avidin has for biotin (Nilson *et al.*, 1997). Further supporting this theory; the first wash released the recombinant protein in addition to *E. coli* proteins and the 2 subsequent washes did not reveal any protein. The 3 elutions released some protein; however, none of the bands corresponded to the molecular weight of the recombinant BFDV capsid protein, indicating that the resin retained non-biotinylated protein during wash steps, but not the biotinylated protein. Thus the protein of interest appears to have bound to the resin at very low level, and the portion of recombinant protein that may have bound was removed already in the first wash.

Purification attempts using the column capture method gave similar results, except a minute amount of the recombinant protein was observed in the elutions. The elutions from the resin were contaminated with *E. coli* proteins, but did have considerably less *E. coli* protein content than can be seen in non purified protein preparations. Thus the column method allowed for more binding between avidin and biotin than the batch method. The primary culture used in expression experiments was not supplemented with biotin, despite the direction of the manufacturer, because the presence of biotin in growth media was found to inhibit growth. The lack of biotin in primary culture would have caused the biotin ligase pathway to not be primed before the culture was diluted and recombinant protein expression induced. The result of this would be that fewer cells were able to perform biotinylation and therefore lowering the yield of expressed

biotinylated protein and further less protein would be available for binding to the avidin linked resin. Further, this would decrease the ability of biotinylated protein to compete with the non-specific binding activity of *E. coli* proteins, further explaining the high impurity of eluted protein. In addition, biotin not ligated to the recombinant protein as a result of not priming the biotin ligase pathway or a hight ratio of biotin to recombinant protein would result in left over biotin in the lysate. This free biotin would then compete with the recombinant protein for binding sites. Another factor that may affect the binding of the biotinylated protein to the resin is the folding of the protein as this may partially mask the biotin molecule thereby decreasing its ability to bind avidin.

Repeated attempts at using these 2 methods, both according to the directions provided by the manufacturer and several variants of these directions, were unsuccessful at producing entirely pure protein. It can be concluded however that the column capture method allows for more association between biotin and avidin than the batch method, however using this method for purifying recombinant BFDV capsid protein requires more optimisation in order to retain pure recombinant protein.

The reasons for inadequacies of this system when applied to the purification of recombinant BFDV capsid protein are not clear. However, they should be considered an effect of the properties of this recombinant protein and not a poorly designed purification system, due to successful use of this system for producing and purifying other proteins previously by this laboratory (unpublished data) and by others (Cress *et al.*, 1993).

4.3.2 *Full length insoluble inclusion body protein*

Insoluble IB protein was purified from crude lysate by low speed centrifugation and solubilised using the Urea/DTT and the B-PER methods. Purification by both methods

reduced the contaminating *E. coli* proteins but some were still evident in the protein preparation. The purified fraction was approximately 20 times more concentrated than the crude lysate, thus a large quantity of *E. coli* contaminants have been removed during the wash steps.

IBs are electron-dense structures that may be observed as refractile bodies under phase contrast microscopy. These structures appear as amorphous particles under electron microscopy and there have been no reports of regular structure (Chrunyk *et al.*, 1993). IBs were first observed in bacteria that globally missincorporated a.a. analogues into their proteins. Later, such aggregates have been observed in many recombinantly expressed proteins (Chrunyk *et al.*, 1993). Problems associated with protein solubility are often described in the literature; however no exact methods of solving these problems have been determined.

High level expression of cloned genes in *E. coli* may result in the protein forming IB aggregates. Several factors may contribute to the fate of a recombinant protein as an IB, including host cell, growth conditions and properties of the protein of interest. (Kane and Hartley, 1998) reviewed an extensive list of proteins originating from *E. coli*, *Bacillus* species, Cellulomonas, viral, mammalian and *S. cerevisiae* expressed in *E. coli* systems, and report that there seems to be no correlation between formation of inclusion bodies and promoter used, formation of disulphide bonds in the recombinant protein, molecular mass of the protein or the level of expression. Normal *E. coli* proteins expressed at high levels may also accumulate in the IB form, and therefore it cannot be assumed that IBs are simply a response to foreign proteins (Marston, 1986). This popular theory of 'response to foreign protein' is further contradicted by the fact that bacteria can package its own proteins into IBs, and no evidence has been reported for IB formation being an active process (Schein, 1989).

Several reasons have been postulated for why IBs occur. One of these is that proteins aggregate when they are expressed at a level that saturates the cell's degradation system. However, there are some proteins, including prochymosin which are also insoluble when produced at low levels. It does seem evident that IBs are not the sole product of precipitation, as solubilisation of recombinant protein IBs requires harsh chemical conditions, thus at some stage covalent, ionic or hydrophobic interactions must occur between the molecules in IBs (Marston, 1986). Schein (1988) expressed 3 different proteins in *E. coli* at 37°C and also at temperatures ranging between 23 and 30°C. When expressed at 37°C all 3 proteins were found as IB aggregates, however at temperatures between 23 and 30°C, 30-90% of the recombinant protein was found in the soluble form. These effects were observed for several different *E. coli* strains and plasmid constructs. The same results have been found with the P22 tailspike protein, diphtheria toxin, ricin A chain and several others (Schein, 1989). Thus temperature appears to be a major determinant of the solubility of foreign expressed proteins in *E. coli*. Expression of interferon-α2 in *E. coli* has shown that the percentage of insoluble protein does not correlate with the total amount of protein expressed (Schein, 1989). Others have demonstrated mutants that produce *less* inclusion IBs and mutants that produce *more* IBs than the wild type, indicating that a.a. sequence may influence the level of formation of IBs (Wetzel *et al.*, 1991; Chrunyk *et al.*, 1993).

Thus the exact mechanism of IB formation has not been elucidated. However, complications of IB occurrence may be overcome by mutations, altering temperature at which the protein is expressed and in some cases by decreasing the level of expression. For this experiment, attempting expression at lower temperatures would have been the next step in the effort to produce a higher soluble fraction of full length and C-terminal truncated protein. This was not performed due to limited time and the fact that expressed soluble protein was not considered Alfa Omega to big picture of this project.

IBs are dense and therefore sediment more rapidly than cellular debris during low speed centrifugation, a property that may be exploited for purification of IB protein (Marston, 1986). Centrifugation speeds ranging from 500 to 12,000 *g* have been reported for purification of inclusion bodies (Marston, 1986). In this experiment initial centrifugation of cell lysate was performed at 2,000 *g* for 7 min as described in section *2.8.2*. Considering the broad range of centrifugal speeds that have been reported for IB purification purposes, 2,000 *g* may not have been the optimal, and purification could possibly have been enhanced by either altering the speed or the centrifugation time, or a combination of both. Impurity of the final product may also be explained by the nature of IBs, because they commonly contain other components such as the 4 subunits of RNA polymerase, some of the bacterial outer membrane proteins (OmpC, OmpF and OmpA), 16S and 23S rRNA, and possibly circular and nicked plasmid DNA in addition to the recombinant protein (Schein, 1989).

4.4 Detection of full length recombinant protein with anti BFDV specific antibodies

Full length recombinant BFDV capsid protein was successfully recognised by sera from a naturally immune cockatoo and by sera from a chicken experimentally challenged with live BFDV. There was no reaction with naïve chicken sera, confirming that the antibody-antigen reaction seen with challenged chicken sera in fact was interaction between chicken-anti-BFDV-antibody and the recombinant protein.

These results show that specific anti-BFDV-antibodies recognise the recombinant protein even though the protein was solubilised in 2M Urea, thus the linear epitopes involved in the animal antibody response have been conserved in the recombinant protein.

4.5   Detection of antibody-response to recombinant BFDV protein in sheep and psittacine birds

The antibody response to inoculation with the recombinant protein was detected in sheep by western immunoblot and IHC and in psittacine birds by HI. The results for inoculated sheep will be discussed in sections 4.5.1 and 4.5.2 and results regarding vaccination of psittacine birds will be discussed in section 4.5.3.

4.5.1   Western immunoblot detection of antibodies to full length recombinant BFDV capsid protein in sheep

Western immunoblot with sera from the 2 sheep, both bled and inoculated at days 0, 21 and 44, successfully detected antibodies to the recombinant BFDV protein. These antibodies were not present before inoculation, but a strong signal was observed in sera from 21 days post inoculation. An even stronger signal was observed with sera collected 24 days post boosting (44 days post primary inoculation), showing as expected that boosting increases the antibody response. These results prove that the recombinant BFDV capsid protein induces an antibody response. Under optimal circumstances, this western immunoblot would have employed native virus in place of the recombinant protein, thereby determining whether these antibodies would recognise native virus. This was not possible due to the limited availability of purified virus, as the virus cannot be propagated in cell culture. However this uncertainty was clarified by IHC as discussed below.

4.5.2   IHC detection of antibodies to native BFDV raised in sheep inoculated with recombinant BFDV capsid protein

Screening inoculated sheep sera by IHC successfully demonstrated the ability of sera raised against the recombinant protein to recognise BFDV inclusions in skin sections

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from a PBFD-affected cockatoo. Formation of antibody-antigen complexes was not detected with non-inoculated sheep sera, confirming that the antibody-antigen reaction observed with post inoculation sera was a result of sheep-anti-recombinant BFDV Protein-antibodies recognising native BFDV. This proves that inoculation with the recombinant protein induces an antibody response that also recognises native virus, giving the first evidence for the effectiveness of this bacterially expressed recombinant protein for use in vaccination to protect psittacine birds against BFDV.

4.5.3 HI detection of antibodies to native BFDV raised in psittacine birds vaccinated with recombinant BFDV capsid protein

HI successfully detected and quantified an increase in antibody titre towards native BFDV in 3 of 12 vaccinated birds. Two of these birds did not have a HI detectable anti-BFDV-antibody titre before vaccination. These results do not necessarily mean there was no antibody response to the vaccine in the remaining 10 birds. Some sera caused low levels of auto-agglutination when screened, interfering with the principles of the assay. Therefore the antibody titre of these sera could not be determined. The HI assay is not a sensitive technique, the first well in a row being a 1:20 dilution of sera. Therefore a low antibody titre would not be detected by HI. Inability to produce HI detectable antibody titres in some individuals may also be due to no previous exposure to the virus, thus a higher vaccine dose might be required to induce a HI detectable antibody response. Some birds became unavailable for boosting and/or bleeding at various times post vaccination (as indicated by "N/A" in results table). Therefore some birds may not have produced HI detectable antibody, as vaccination commonly requires more than one injection for inducing a high level antibody response at a high level and some birds may have increased their HIU status but were not detected because of lacking access to the animal.

A HI assay for the detection of psittacine-anti-BFDV-antibody has been optimised (Riddoch *et al.*, 1996) and has become the standard for detecting and quantifying antibody to BFDV in psittacine birds. This assay was successfully applied for quantification of antibody response in the experimental birds, however the assay was found insufficient for quantifying antibody response in experimental sheep, as the sheep sera caused low levels of auto-agglutination which interfered with the principles of the HI test.

4.6   Future directions

Before this recombinant protein can be used to vaccinate endangered species, further investigations are required as follows:

- Developing an efficient method to increase the volume of production of recombinant protein.

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

- Vaccination trials to determine the minimal effective vaccine dose of recombinant protein that will induce a persistent effective immunity. This will include determining the number of doses required and the optimal time between doses.

  o Given the low sensitivity of HI for detecting circulating anti-BFDV antibodies in blood, the development of more sensitive serological assays (such as ELISA) will be required.   The production of specific monoclonal antibodies will be a necessary element in the development of a serological assay.

o A better understanding of the antigenic variation that exists among BFDV isolates from wild endangered psittacine bird species will also be required to ensure that any vaccine developed is efficacious against all antigenic "serotypes".

- Additionally, experimentally vaccinated birds have to be challenged with live virus to determine whether vaccination confers immunity to BFDV infection. This will require the development of specific pathogen free flocks (SPF) of psittacine birds that can act as experimental models of infection.

- Determining the level of passively transferred immunity that occurs between parent (hen) and offspring via the egg yolk will facilitate protection of nestling birds.

- Developing novel techniques that can facilitate the safe and efficacious vaccination of free-living wild birds with minimum impact on individual birds and the environment should also be considered.

Future work required		
Action	Priority	Indicative cost estimate
Increasing production volume and purification of recombinant protein	Medium	$20-30,000
Development of more specific and sensitive antibody tests (such as ELISA) for BFDV antibody	High	$174,000
Production of monoclonal antibodies to BFDV (to facilitate the construction of sensitive and specific diagnostic assays)	High	$48,000
Improved understanding of the antigenic and genotypic variation of BFDV, particularly in endangered birds	Medium	$80,000
Develop an SPF flock that can be used for vaccination response and protection experiments	High	$170,000
Vaccination dose, challenge and effectiveness experiments	High	$180,000
Development of novel "minimum interference" techniques for administering vaccine to wild birds	Medium	$240,000
Environmental impact assessment of vaccination	High	$50,000

Conclusions

Expression of full length and C-terminal truncated BFDV capsid protein was successfully optimised. The full length protein was proven to be recognisable by sera raised against native virus in naturally infected psittacine birds, as well as experimentally infected chicken. Sera from a chicken that had not been challenged did not recognise the recombinant protein showing that the antibody-antigen complex formation seen with the challenged chicken sera was a result of anti-BFDV-antibodies recognising the recombinant protein. This shows that the antigenic epitopes of the native virus have been conserved in the recombinant protein. Further, the recombinant

protein was proven to be antigenic by inoculating sheep and detecting antibody response by western immunoblot. Pre inoculation sera did not display reactivity with the recombinant protein. The antisera raised in inoculated sheep was further proven by IHC to react with native BFDV, proving that inoculation with the recombinant protein induced an antibody response that recognises native virus. Finally, vaccination of psittacine birds with the recombinant protein resulted in an antibody response that can be detected by HI.

5   References

Almeida, M. S., Torloni, H., Lee-Ho, P., Vilar, M. M., Thaumaturgo, N., Simpson, A. J. and Tendler, M. (2003). Vaccination against Fasciola hepatica infection using a Schistosoma mansoni defined recombinant antigen, Sm14. Parasite Immunol, 25, 135-7.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nuc Acids Res, 25, 3389-3402.

Applied Biosystems (1995-1998). ABI Prism Primer Express, version 1.0

Arguello-Astorga, G., Herrera-Estrella, L. and Rivera-Bustamante, R. (1994). Experimental and theoretical definition of geminivirus origin of replication. Plant Mol Biol, 26, 553-6.

Ashby, E. (1907). The Emu, 6, 193-194.

Bassami, M. R., Berryman, D., Wilcox, G. E. and Raidal, S. R. (1998). Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virol, 249, 453-9.

Bassami, M. R., Ypelaar, I., Berryman, D., Wilcox, G. E. and Raidal, S. R. (2001). Genetic diversity of beak and feather disease virus detected in psittacine species in Australia. Virol, 279, 392-400.

Chrunyk, B. A., Evans, J., Lillquist, J., Young, P. and Wetzel, R. (1993). Inclusion body formation and protein stability in sequence variants of interleukin-1 beta. J Biol Chem, 268, 18053-61.

Cobb, B. D. and Clarkson, J. M. (1994). A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. Nuc Acids Res, 22, 3801-3805.

Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. and Wingfield, P. T. (Eds.) (2002) Curr Prot Protein Sci, John Wiley and Sons.

Cress, D., Shultz, J. and Breitlow, S. (1993) In Promega Notes Magazine, pp. 2-9.

Engel, B. (1992). Amplify for Analysing PCR Experiments. University of Wisconsin, USA.

Gerlach, H. (1994) In Avian Medicine: Principles and Application (Eds, Ritchie, B. W., Harrison, G. J. and Harrison, L. R.) Wingers Publishing, Lake Worth, Florida, pp. 894-903.

Gronenborn, B. (2004). Nanoviruses: genome organisation and protein function. Vet Microbiol, 98, 103-9.

Gutierrez, C., Ramirez-Parra, E., Mar Castellano, M., Sanz-Burgos, A. P., Luque, A. and Missich, R. (2004). Geminivirus DNA replication and cell cycle interactions. Vet Microbiol, 98, 111-9.

Hodgeman, T. C. (1988). A new superfamily of replicative proteins. Nat, 333, 22-23.

Jacobson, E. R., Clubb, S., Simpson, C., Walsh, M., Lothrop, C. D., Jr., Gaskin, J., Bauer, J., Hines, S., Kollias, G. V., Poulos, P. and et al. (1986). Feather and beak dystrophy and necrosis in cockatoos: clinicopathologic evaluations. J Am Vet Med Assoc, 189, 999-1005.

Jergens, A. E., Brown, T. P. and England, T. L. (1988). Psittacine beak and feather disease syndrome in a cockatoo. J Am Vet Med Assoc, 193, 1292-4.

Johne, R., Raue, R., Grund, C., Kaleta, E. F. and Muller, H. (2004). Recombinant expression of a truncated capsid protein of beak and feather disease virus and its application in serological tests. Avian Pathol, 33, 328-36.

Kane, J. F. and Hartley, D. L. (1998). Formation of recombinant protein inclusion bodies in Escherichia coli,. Trends Biotech, 6, 95-101.

Kock, N. D., Hangartner, P. U. and Lucke, V. (1993). Variation in clinical disease and species susceptibility to psittacine beak and feather disease in Zimbabwean lovebirds. Onderstepoort J Vet Res, 60, 159-61.

Latimer, K. S., Steffens, W. L., 3rd, Rakich, P. M., Ritchie, B. W., Niagro, F. D., Kircher, I. M. and Lukert, P. D. (1992). Cryptosporidiosis in four cockatoos with psittacine beak and feather disease. J Am Vet Med Assoc, 200, 707-10.

Law, R. H., Smooker, P. M., Irving, J. A., Piedrafita, D., Ponting, R., Kennedy, N. J., Whisstock, J. C., Pike, R. N. and Spithill, T. W. (2003). Cloning and expression of the major secreted cathepsin B-like protein from juvenile Fasciola hepatica and analysis of immunogenicity following liver fluke infection. Infect Immun, 71, 6921-32.

Liu, C., Ihara, T., Nunoya, T. and Ueda, S. (2004). Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. J Vet Med Sci, 66, 237-42.

Liu, Q., Tikoo, S. K. and Babiuk, L. A. (2001). Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. Virol, 285, 91-9.

Marston, F. A. (1986). The purification of eukaryotic polypeptides synthesized in Escherichia coli. Biochem. J., 240, 1-12.

McOrist, S., Black, D. G., Pass, D. A., Scott, P. C. and Marshall, J. (1984). Beak and feather dystrophy in wild sulphur-crested cockatoos (Cacatua galerita). J Wildl Dis, 20, 120-4.

Meehan, B. M., Creelan, J. L., McNulty, M. S. and Todd, D. (1997). Sequence of porcine circovirus DNA: affinities with plant circoviruses. J Gen Virol, 78 (Pt 1), 221-7.

Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D. and Paul, P. S. (2000). Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol, 81, 2281-7.

Niagro, F. D., Forsthoefel, A. N., Lawther, R. P., Kamalanathan, L., Ritchie, B. W., Latimer, K. S. and Lukert, P. D. (1998). Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. Arch Virol, 143, 1723-44.

Nilson, J., Ståhl, S., Lundberg, J., Uhlèn, M. and Nygren, P. (1997). Affinity Fusion Strategies for Detection, Purification and Immobilization of Recombinant Proteins. Protein Expr and Purif, 11, 1-16.

Pass, D. A. and Perry, R. A. (1984). The pathology of psittacine beak and feather disease. Aust Vet J, 61, 69-74.

Pass, D. A. and Perry, R. A. (1985). Psittacine Beak and Feather Disease: An update. Aust Vet Practit, 15, 55-60.

Promega (2000). Technical Manual, No. 028. Madison, WI, USA, pp. 1-27.

Promega (2001). Technical Bulletin, No. 234. Madison, WI, USA.

Raidal, S. R. (1994). Studies on Psittacine Beak and Feather Disease, PhD thesis, University of Sydney, Sydney.

Raidal, S. R. (1995). Viral Skin Diseases of Birds. Sem Avian and Exotic Pet Med, 4, 72-82.

Raidal, S. R. and Cross, G. M. (1994a). Control By Vaccination of Psittacine Beak and Feather Disease in a Mixed Flock of Agapornis spp. Aust Vet Practit, 24, 178-180.

Raidal, S. R. and Cross, G. M. (1994b). The haemaglutination spectrum of psittacine beak and feather disease virus. Avian Pathol, 23, 621-630.

Raidal, S. R. and Cross, G. M. (1995). Acute Necrotizing Hepatitis Caused by Experimental Infection with Psittacine Beak and Feather Disease Virus. J Avian Med and Surg, 9, 36-40.

Raidal, S. R., Firth, G. A. and Cross, G. M. (1993a). Vaccination and challenge studies with psittacine beak and feather disease virus. Aust Vet J, 70, 437-41.

Raidal, S. R., McElnea, C. L. and Cross, G. M. (1993b). Seroprevalence of psittacine beak and feather disease in wild psittacine birds in New South Wales. Aust Vet J, 70, 137-9.

Raidal, S. R., Sabine, M. and Cross, G. M. (1993c). Laboratory diagnosis of psittacine beak and feather disease by haemagglutination and haemagglutination inhibition. Aust Vet J, 70, 133-7.

Ramis, A., Latimer, K. S., Niagro, F. D., Campagnoli, R. P., Ritchie, B. W. and Pesti, D. (1994). Diagnosis of psittacine beak and feather disease (PBFD) viral infection, avian polyomavirus infection, adenovirus infection and herpesvirus infection in psittacine tissues using DNA in situ hybridization. Avian Pathol, 23, 643-657.

Riddoch, P. A., Raidal, S. R. and Cross, G. M. (1996). Psittacine circovirus antibody detection and an update on the methods for diagnosis of psittacine beak and feather disease. Aust Vet Pract, 26, 134-&.

Ritchie, B. W., Niagro, F. D., Latimer, K. S., Steffens, W. L., Pesti, D., Ancona, J. and Lukert, P. D. (1991). Routes and prevalence of shedding of psittacine beak and feather disease virus. Am J Vet Res, 52, 1804-9.

Ritchie, B. W., Niagro, F. D., Lukert, P. D., Latimer, K. S., Steffens, W. L. and Pritchard, N. (1989a). A Review of Psittacine Beak and Feather Disease; Characteristics of the PGFD Virus. J Wildl Dis, 3, 143-149.

Ritchie, B. W., Niagro, F. D., Lukert, P. D., Steffens, W. L., 3rd and Latimer, K. S. (1989b). Characterization of a new virus from cockatoos with psittacine beak and feather disease. Virol, 171, 83-8.

Ritchie, P. A., Anderson, I. L. and Lambert, D. M. (2003). Evidence for specificity of psittacine beak and feather disease viruses among avian hosts. Virol, 306, 109-15.

Roitt, I., Brostoff, J. and Male, D. (2001) Immunol, Mosby, Edindurgh.

Sambrook, J. and Russel, D. W. (2001) Molecular Cloning a Laboratory Manual, Cold Spring Harbour Laboratory Press, New York.

Schein, C. H. (1989). Production of soluble recombinant proteins in bacteria. Biotech (N Y), 7, 1141-1149.

Schein, C. H. and Noteborn, M. H. (1988). Formation of soluble recombinant proteins in Escherichia coli is favored by lower growth temperature. Biotech (N Y), 6, 291-294.

Schoemaker, N. J., Dorrestein, G. M., Latimer, K. S., Lumeij, J. T., Kik, M. J., van der Hage, M. H. and Campagnoli, R. P. (2000). Severe leukopenia and liver necrosis in young African grey parrots (Psittacus erithacus erithacus) infected with psittacine circovirus. Avian Dis, 44, 470-8.

Sinnathamby, G., Renukaradhya, G. J., Rajasekhar, M., Nayak, R. and Shaila, M. S. (2001). Immune responses in goats to recombinant hemagglutinin-neuraminidase glycoprotein of Peste des petits ruminants virus: identification of a T cell determinant. Vacc, 19, 4816-23.

Studdert, M. J. (1993). Circoviridae: new viruses of pigs, parrots and chickens. Aust Vet J, 70, 121-122.

Tischer, I., Mields, W., Wolff, D., Vagt, M. and Griem, W. (1986). Studies on epidemiology and pathogenicity of porcine circovirus. Arch Virol, 91, 271-6.

Todd, D. (2000). Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathol, 29, 373-394.

van Regelmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. and Wickner, R. B. (Eds.) (2000) Seventh report of the international committee on taxonomy of viruses, Academic Press.

Wetzel, R., Perry, L. J. and Veilleux, C. (1991). Mutations in human interferon gamma affecting inclusion body formation identified by a general immunochemical screen. Biotech (N Y), 9, 731-7.

Wylie, S. L. (1991). Studies on Psittacine Beak and Feather Disease, PhD thesis, Murdoch University, Perth.

Wylie, S. L. and Pass, D. A. (1987). Experimental reproduction of psittacine beak and feather disease/french moult. Avian Pathol, 16, 269-281.

Ypelaar, I., Bassami, M. R., Wilcox, G. E. and Raidal, S. R. (1999). A universal polymerase chain reaction for the detection of psittacine beak and feather disease virus. Vet Microbiol, 68, 141-8.

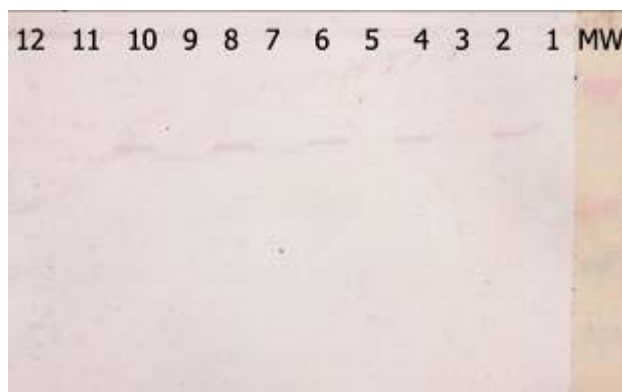# Appendix A. Optimisation of full length protein expression

Figure A.1. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 0.32. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG.
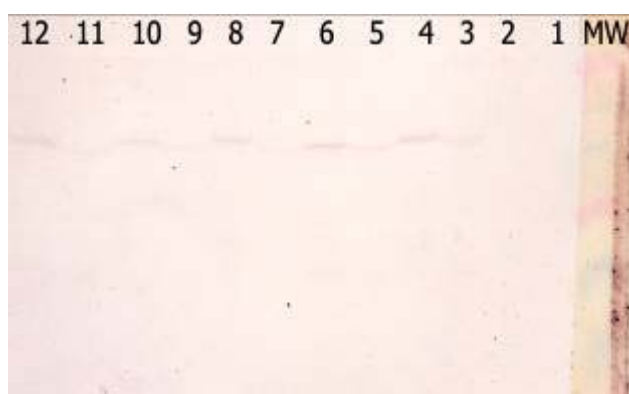

Figure A.2. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 0.32. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 and 6 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 and 6 hours of induction with IPTG.
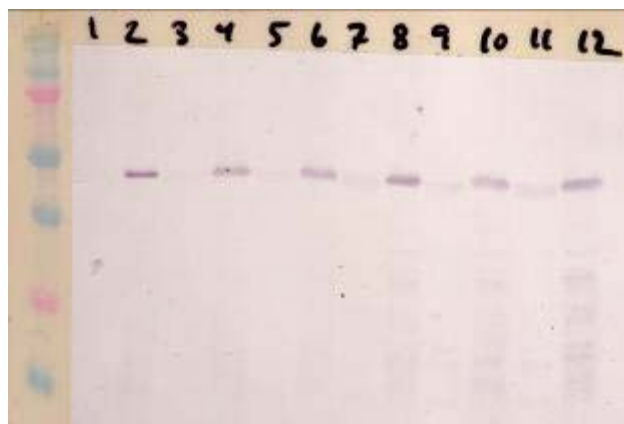
Figure A.3. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.62. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG.

Figure A.4. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.62. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

Figure A.5. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.90. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9, 11 and 13: Soluble protein fractions before induction, after 0.5, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10, 12 and 14: Insoluble protein fraction before induction, after 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

Figure A.6. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.90. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, and 9: Soluble protein fractions after 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8 and 10: Insoluble protein fraction after 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.
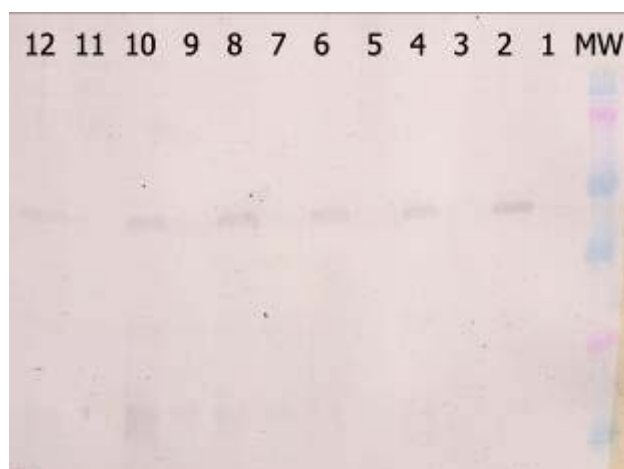
Figure A.7. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 1.19. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG.
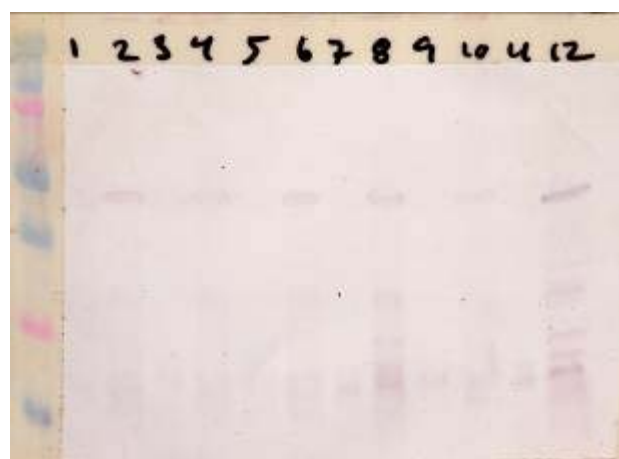

Figure A.8. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 1.19. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

Appendix B. Optimisation of C-terminal truncated protein
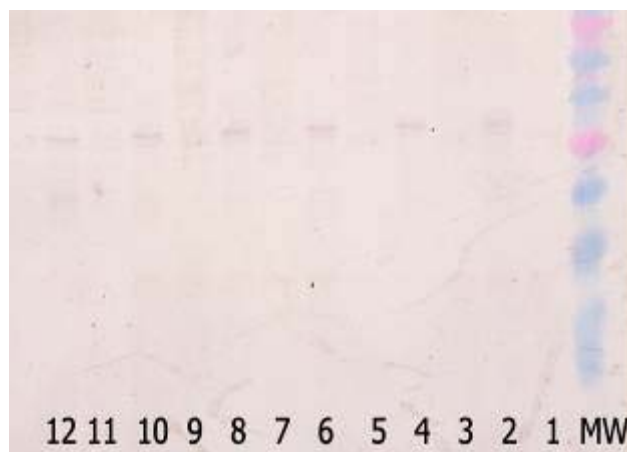
expression

Figure B.1. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.316. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fraction after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively.
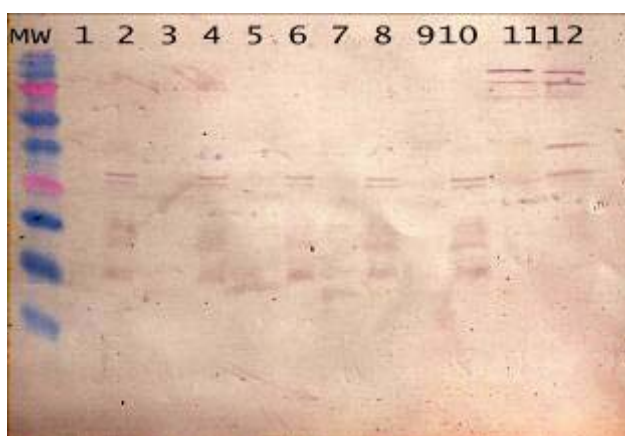

Figure B.2. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.316. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

Figure B.3. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.591. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fraction after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively.

Figure B.4. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.591. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.
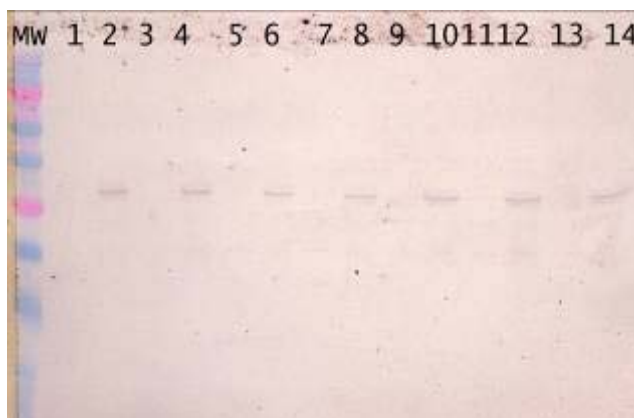
Figure B.5. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.940. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fraction after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively.

Figure B.6. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.940. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

Figure B.7. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 1.174. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively
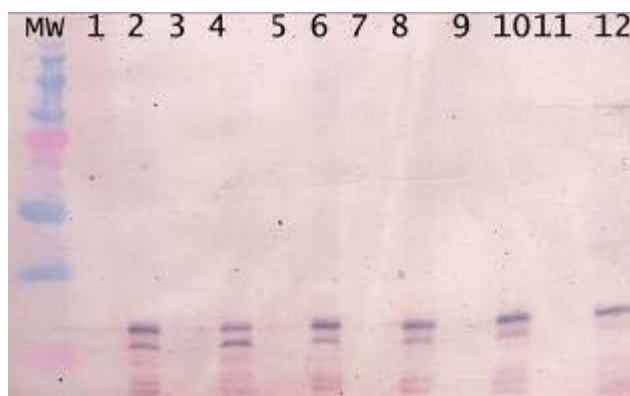

Figure B.8. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 1.174. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.