Establishment of Serological and Molecular Techniques to Investigate Diversity of Psittacine Beak and Feather Disease Virus in Different Psittacine Birds in South Africa.

By

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Submitted in accordance with the requirements for the degree of

Magister Scientiae

In the

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November 2004

This dissertation is dedicated to my husband, Lloyd, my parents, Mr. Mohamedali and Mrs. Wijitha Tajbhai and my family.

ACKNOWLEDGEMENTS.

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LIST OF ABBREVIATIONS.

^oC	Degrees Celsius
λ	Lambda phage DNA
$\mu g.ml^{-1}$	Microgram per milliliter
µl	Microliter
µm	Micrometer
µM	Micromolar
ρmoles	Picomoles
A_{450}	Absorbance at 450 nanometers
AGP	African grey parrot
APV	*Avian polyoma virus*
b	Bases
BBTV	*Banana bunchy top virus*
BFDV	*Beak and feather disease virus*
BG*	Budgerigar
BHP	Brown headed parrot
bp	Base pairs
BPL	β-Propiolactone
C	Complimentary sense
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CAV	*Chicken anaemia virus*
CFDV	*Coconut foliar decay virus*
CK*	Cockatoo
cm	Centimeter
cntd	Continued
CoCV	*Columbid circovirus*
CP	Coat protein
CsCl	Caesium chloride
DNA	Deoxyribonucleic acid

LIST OF ABBREVIATIONS.

dNTP	Deoxyribonucleotide triphosphate
DOE-Vacc	Double-oil emulsion adjuvant vaccine
ds	Double stranded
EC	Eastern Cape province
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FCA	Freund's complete adjuvant
FEB	February
FIA	Freund's incomplete adjuvant
FS	Free State province
G	Gauteng province
GA	Glutaraldehyde
$g.ml^{-1}$	Gram per milliliter
GTE	Glucose-Tris-EDTA
HA	Haemagglutination assay
HCl	Hydrochloric acid
HI	Haemagglutination inhibition
H_2O_2	Hydrogen peroxide
H_3PO_4	Orthophosphoric acid
H_2SO_4	Sulphuric acid
ICTV	International Committee on Taxonomy of Viruses
IM	Intramuscular
IPTG	Isopropyl-2-D-thiogalactopyranoside
JAN	January
KAc	Potassium acetate
kb	Kilobases
KCl	Potassium chloride
kDa	KiloDaltons
KH_2PO_4	Potassium dihydrogen orthophosphate

LIST OF ABBREVIATIONS.

KOH	Potassium hydroxide
KZN	Kwa Zulu Natal province
LB*	Lovebird
LB	Luria Bertani
LK*	Lorikeet
LP	Limpopo province
Mg^{2+}	Magnesium ions
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
$mg.ml^{-1}$	Milligram per milliliter
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
M	Molar
M_r/MW	Molecular weight
MWCO	Molecular weight cut off
N	Normality
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium hydrogen carbonate
NaH_2PO_4	Sodium dihydrogen orthophosphate
Na_2HPO_4	Disodium hydrogen orthophosphate
NaOH	Sodium hydroxide
Na_3PO_4	Trisodium orthophosphate
N-J	Neighbour-joining
nm	Nanometers
no	Number
NOV	November
nt	Nucleotides

LIST OF ABBREVIATIONS.

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBFD	Psittacine beak and feather disease
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	*Porcine circovirus*
PEG	Polyethylene glycol
PMWS	Post-weaning multisystemic wasting syndrome
RBCs	Red blood cells
RCR	Rolling circle replication
Rep	Replication associated
RF	Replicative form
RFLP	Restriction length fragment polymorphism
RNA	Riboxynucleic acid
rpm	Revolutions per minute
rt	Room temperature
s	Seconds
SCSV	*Subterranean clover stunt virus*
SDS	Sodium dodecyl sulphate
SEPT	September
ss-DNA	Single stranded DNA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TLMV	*TTV-like mini virus*
TMB	4,4,5,5'-Tetramethylbenzidine
Tris	Tris (hydroxymethyl) aminomethane
TST	Tris-Sodium chloride-Tween 20
TTV	*TT virus*
UFS	University of the Free State

LIST OF ABBREVIATIONS.

USA	United States of America
UV	Ultraviolet
v	Virus sense
v/v	Volume per volume
WC	Western Cape province
w/v	Weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-A-D-galactopyranoside

CHAPTER 1.

LITERATURE REVIEW.

1.1: INTRODUCTION.

Porcine circovirus (PCV), a contaminant in the continuous pig kidney cell-line PK-15, was first encountered in Germany in 1974 (Studdert, 1993; Todd, 2000; Maramorosch *et al.*, 2001) and was the first demonstration of an animal circovirus. The second circovirus to be discovered was *Psittacine beak and feather disease virus* (BFDV) followed by *Chicken anaemia virus* (CAV) which was first identified in Japan in 1979 as a cause of increased mortality associated with anaemia, lymphoid depletion, liver changes and haemorrhages in chickens (Studdert, 1993).

Psittacine beak and feather disease (PBFD) is a common dermatologic condition in parrots caused by BFDV (Schoemaker *et al.*, 2000) that was first discovered in 1975 in cockatoos in Australia. However, it is possible that PBFD had been noticed as early as 1887 by Australian explorers who described characteristic feather changes in free-ranging Red-rumped parrots (*Psephotus sp.*) in South Australia (Ritchie & Carter, 1995).

1.2: TAXONOMY.

The 6th report of the International Committee on Taxonomy of Viruses (ICTV) classified CAV, PCV and BFDV into the *Circovirus* genus within the family *Circoviridae* with CAV being designated the type species. Virions in the family *Circoviridae* are non-enveloped icosahedrons, 17-22 nm in diameter (Murphy *et al.*, 1995) with covalently closed, circular, negative-sense, single-stranded DNA (ss-DNA) genomes between 1.7-2.3 kilobases (kb) in size. Based on their circular, ss-DNA genomes (~1 kb) and their icosahedral capsids, three plant

pathogens namely *Coconut foliar decay virus* (CFDV), *Banana bunchy top virus* (BBTV) and *Subterranean clover stunt virus* (SCSV), were classified as unassigned viruses of *Circoviridae*.

In the current 7th ICTV report, the plant viruses have been reclassified into the newly established plant *Nanovirus* genus. With the recognition of two types of PCV: PCV-1 which is non-pathogenic and PCV-2 which is associated with post weaning multisystemic wasting syndrome (PMWS) in pigs (Todd, 2000) as well as the differences between CAV genome expression and that of PCV and BFDV, the family *Circoviridae* has been divided into two separate genera: *Circovirus* and *Gyrovirus*. PCV-1, PCV-2 and BFDV are classified in the genus *Circovirus* with PCV being the type species while CAV belongs to the genus *Gyrovirus* and is the type species for the genus.

The increasing identification of new avian circoviruses has led to the identification and placement of *Columbid circovirus* (CoCV) and *Goose circovirus* as tentative members of the *Circovirus* genus (Eisenberg *et al.*, 2003). Based on homology studies with the viral genomes, *Canary circovirus* can also be grouped in this genus (Raue *et al.*, 2004).

The *TT virus* (TTV) which may be the first human circovirus identified was recognized in the serum from a Japanese patient (initials T.T.) with post-transfusion hepatitis (Todd, 2000). TTV and the *TTV-like mini virus* (TLMV) share similarities in genome organization with CAV and their taxonomic position is still under consideration (Raue *et al.*, 2004) although a new virus family with the name *Circinoviridae* has already been proposed.

1.3: VIRUS ASPECTS.

1.3.1: BIOLOGICAL CHARACTERISTICS.

1.3.1.1: Morphology.

BFDV has a non-enveloped, icosahedral or spherical capsid (Figure 1.1) which is about 20% smaller than CAV, with no obvious surface structure (Maramorosch *et al.,* 2001). Its diameter ranges between 14 and 17 nanometres making it one of the smallest animal viruses.
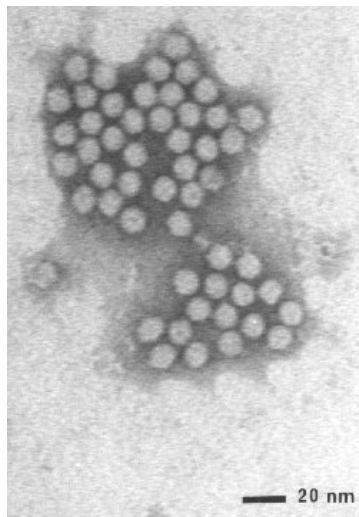
Figure 1.1: Electron micrograph of negatively stained BFDV particles http://numbat.murdoch.edu.au/caf/pbfd.htm.

Intranuclear and intracytoplasmic basophilic inclusions have been identified in follicular epithelial cells and in macrophages of the feather pulp respectively. Other tissues where viral inclusions have been observed include the beak and palate, bursa of Fabricius, thymus, tongue, parathyroid gland, crop, oesophagus, spleen, intestines, bone marrow, liver, thyroid, testis, ovary and adrenal glands (Ramis *et al.,* 1998).

Electron microscopically, intracytoplasmic inclusions have been reported to be composed of electron-dense granules 17-22 nm in diameter (Ritchie *et al.*, 1989) that form paracrystalline arrays, semicircles, concentric circles, whorls and other configurations (Trinkaus *et al.*, 1998; Sanada *et al.*, 1999). Viruses like *Avian polyoma virus* (APV) and *Adenovirus* produce similar basophilic nuclear inclusions to that of BFDV making it difficult to diagnose BFDV infection based solely on histopathology thus dictating the use of additional diagnostic tests.

1.3.1.2: Isolation.

Of the four classified circoviruses, only CAV and PCV have been propagated in cell culture (Maramorosch *et al.*, 2001). So far all attempts to cultivate BFDV have been unsuccessful and a cell culture system in which the virus will persist for more than several passages has yet to be identified (Ritchie *et al.*, 1991a; Todd, 2000; Raue *et al.*, 2004).

Although BFDV has not been grown in cell culture, significant amounts of virus can be purified from feather follicle tracts which has paved the way for the development of a haemagglutination assay (HA) and haemagglutination inhibition (HI) assay for the detection of viral antigen and antibody, respectively (Studdert, 1993).

1.3.1.3: Haemagglutination.

BFDV has agglutinating activity with erythrocytes from a range of psittacine species as well as erythrocytes from geese (*Anser anser*) [Sexton *et al.*, 1994] and guinea pigs (Ritchie *et al.*, 1991b). Psittacine species known to have haemagglutinating activity with BFDV include Goffin's Cockatoo (*Cacatua goffini*), Galah (*Eolophus roseicapillus*), Eastern slender-billed Corella (*Cacatua tenuirostris*), Sulphur crested Cockatoo (*Cacatua galerita*), Gang Gang Cockatoo (*Callocephalon fimbriatum*) and Major Mitchell's Cockatoo (*Cacatua leadbeateri*)

[Soares *et al.*, 1998; Sanada & Sanada, 2000). However, some of the sources of erythrocytes have been reported contrarily and differences in the agglutinating ability of erythrocytes collected from different individuals of the same species have also been reported (Raue *et al.*, 2004).

1.3.1.4: Host Range.

BFDV occurs only in psittacine species with over 40 different species being affected. The disease has been reported in captive and free-ranging Old World psittacines as well as New World Psittaciformes (Ritchie *et al.*, 1992a) although the exact host range is unknown. Species that have been identified with PBFD include Sulphur-crested Cockatoo (*C.galerita*), Major Mitchell's Cockatoo (*C.leadbeateri*), Galah (*C.roseicapilla*), Budgerigar (*Melopsittacus undulatus*), Cockatiel (*Nymphicus hollandicus*), Rainbow Lorikeet (*Trichoglossus haematodus*), Mallee ring-neck parrot (*Barnardius barnardi*), Eclectus parrot (*Eclectus roratus*) [Ritchie *et al.*, 1990] among numerous others. Eisenberg *et al.* (2003) also reported the occurrence of BFDV in ostriches (*Struthie camelus*).

1.3.2: BIOPHYSICAL AND BIOCHEMICAL PROPERTIES.

The biophysical and biochemical properties of CAV, PCV-1 and BFDV are represented in Table 1.1. The inability to propagate BFDV *in vitro* led to purification of the virus from infected feather pulp for its physical characterization. Comparative electron microscopy studies indicate that PCV-1 and BFDV are almost identical in size but 20% smaller than CAV (Todd, 2000; Maramorosch *et al.*, 2001). BFDV also lacks the surface structure observed in CAV particles and has a buoyant density of 1.37 g.ml^{-1} in caesium chloride (CsCl). Polyacrylamide gel electrophoresis (PAGE) has revealed three major proteins associated with BFDV with molecular weights of 26.3 kiloDaltons (kDa), 23.7 kDa and 15.9 kDa (Table 1.1).

The environmental stability of BFDV is unknown although Wylie & Pass (1987) demonstrated the inactivation of the virus by β-propriolactone (BPL). CAV and PCV-1 are highly stable; the cell culture infectivity of each virus was found to resist incubation at 70 ^{o}C for 15 minutes and treatment at pH 3 (Todd, 2000; Maramorosch *et al.*, 2001). In liver tissues CAV remained infectious when treated with amphoteric soap (10%), orthodichlorobenzene (10%), iodine (1%), sodium hypochlorite (1%, bleach), methyl alcohol, ethyl alcohol, chloroform and heating to 80 ^{o}C for one hour, and even when boiled for five minutes. Treatment with ethylene oxide for two hours left dried material containing CAV still infectious while a 24 hour fumigation with formaldehyde only partially inactivated the virus (Ritchie & Carter, 1995).

Table 1.1: Physical and chemical characteristics of CAV, PCV-1 and BFDV (Todd, 2000).

	CAV	PCV-1	BFDV
Particle size (nm)	19.1-26.5	16.8-20.7	14-20.7
Buoyant density (g.ml^{-1} in CsCl)	1.33-1.37	1.36-1.37	1.378
Sedimentation coefficient	91S	57S	-
Genome size (b)	2298/2319	1759	1993-2018
Virion proteins (M_r)	50 000	36 000	26 300, 23 700, 15 900

The ability of BFDV to agglutinate erythrocytes after incubation at 80 ^{o}C for 30 minutes remained unaffected. Further investigations with BFDV have not been possible due to its inability to grow *in vitro* but its similarity in ultrastructure and DNA composition to CAV suggests that it is also highly environmentally stable.

1.3.3: GENOME ORGANISATION.

With the exception of TTV, circoviruses are the only animal viruses that possess circular, ss-DNA genomes that are also the smallest genomes of the animal virus families (Maramorosch *et al.*, 2001). Presently, more than 11 complete genome

sequences of BFDV are available in the NCBI genome database; BFDV's genome consists of between 1992-2018 nucleotides (nt).

Studies on RNA extracted from feather pulp of infected birds revealed the synthesis of transcripts on both the replicative form (RF) strands confirming that like PCV-1 and PCV-2, BFDV possesses an ambisense genome organization. The BFDV genome contains seven major open reading frames (ORFs) [Figure 1.2] that can encode proteins of >8.7 kDa; three ORFs in the encapsidated or virus (V) sense strand and four ORFs in the complementary (C) sense strand of the RF. The genome also lacks a distinct non-coding region (Bassami *et al.*, 1998).
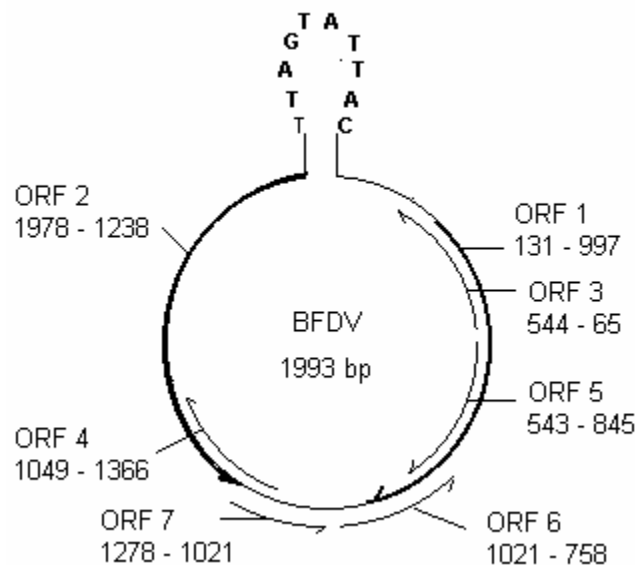
Figure 1.2: A representation of the circular ss-DNA genome of BFDV showing the position of the conserved nonanucleotide motif (TAGTATTAC) and the seven ORFs. Adapted from Bassami *et al.* (1998).

Although up to seven ORFs can be detected in the BFDV genome, reports by Bassami *et al.* (2001) indicated that not all these ORFs are present in all isolates and that ORFs 1, 2 and 5 were conserved among all the isolates. ORF1 or V1 ORF has the most degree of conservation and is known to encode the replication-associated (Rep) protein required for rolling circle replication (RCR),

ORF2 or C1 ORF encodes the capsid protein while ORF5 is thought to encode a protein whose function is as yet unknown.

Another common feature of the PCV and BFDV genomes is the location of a potential stem-loop structure which contains, at its apex, a conserved nonanucleotide motif between the start sites of the V1 and C1 ORFs (Todd *et al.*, 2001). This nonanucleotide motif has the sequence TAGTATTAC in BFDV (Figure 1.2) and is also conserved among plant geminiviruses, plant nanoviruses and bacteriophages like φX174 (Todd, 2000).The convention of numbering the nucleotides was adopted from that used for geminiviruses in which the A residue immediately downstream of the putative nick site (at the position TAGTATT^AC) in the nonanucleotide motif was designated nucleotide position 1 (Bassami *et al.*, 2001).

1.3.4: GENETIC DIVERSITY.

Using ultrastructural characteristics, protein composition and antigenic comparisons, Ritchie *et al.* (1990) studied BFDV isolated from four different psittacine species and concluded that all four isolates were identical. Therefore, it has been assumed that there is only one genetic strain of BFDV worldwide.

More recent studies on Australian BFDV isolates by Bassami *et al.* (2001) indicate that the genetic diversity is higher than was initially suggested. The variable size in genomes is due to small insertions or deletions within non-coding as well as coding (ORF1 and 2) regions whose significance is unknown. The overall nucleotide identity ranged from 84% to 97% although there was no evidence that distinctly different genotypes occurred.

Upon phylogenetic analysis of the predicted amino acid sequences for the Rep protein and capsid protein, isolates were grouped into clusters: one cluster included most isolates but five isolates clustered separately. Ritchie *et al.* (2003)

also analyzed isolates from New Zealand and Australia using the Rep protein sequences to determine if a phylogenetic relationship exists between virus genotypes and host species. Three ancestral lineages were observed: a cockatoo, budgerigar and lorikeet lineage.

Raue *et al.* (2004) found no relationship between individual psittacine species and distinct nucleotide sequences encoding part of the capsid protein. However, in their study of the C1 ORF obtained from an outbreak of acute PBFD, lorikeets (*Trichoglossus sp.*) and African grey parrots (*Psittacus erithacus*) with typical feather disorders clustered in separate branches of a phylogenetic tree. These results could be an indication of the existence of BFDV genotypes in individual psittacine species (Raue *et al.*, 2004).

Heath *et al.* (2004) studied PBFD within southern Africa and found a similar level of genetic diversity in BFDV isolates to that described in Australia and New Zealand. However, their study that involved the phylogenetic analysis of the BFDV coat protein revealed eight lineages in southern Africa with the apparent divergence of the southern African isolates from similar viruses worldwide. These isolates clustered into three unique genotypes with the level of genetic variations being attributed to point mutations and recombination events. A study by Albertyn *et al.* (2004) using restriction fragment length polymorphism (RFLP) analysis of ORF1 in South African BFDV isolates demonstrated the occurrence of different RFLPs which might be an indication of more than one pathogenic strain and further work of this possibility is warranted. Alternatively, Ritchie *et al.* (2003) suggested that all variants of BFDV might infect all psittacine species, but only certain genotypes are pathogenic in a species group.

1.3.5: GENOME REPLICATION.

Circoviruses are highly dependent on cellular enzymes for replicating their DNA due to their limited coding capacities. In the attempt to elucidate the replication

strategies of circoviruses, studies with bacteriophages and plant geminiviruses have proven useful due to structural and genome sequence similarities as well as the presence of conserved DNA and protein sequence motifs in their genomes with that of circoviruses. Circular double-stranded (ds) RF DNAs are essential intermediates in both the transcription and replication of circovirus genomes (Maramorosch *et al.*, 2001). Synthesis of the C strand to generate the first RF forms the first step with further DNA replication progressing by RCR.

Bacteriophage φX174 is used as a replication model for BFDV genome replication. The A-protein cleaves the virus strand DNA at a unique site producing a 3'-OH terminus (which acts as a primer) that is extended by a cellular DNA polymerase. As elongation proceeds, the virus strand is displaced after which the A-protein cleaves it from the newly synthesized strand and self ligates it to form circular ss-DNA (Maramorosch *et al.*, 2001). These strands can either be encapsidated into virus particles or serve as templates for complementary strand synthesis to generate more RFs.

The Rep protein in geminiviruses serves the same purpose as the A-protein and its similarity to that of BFDV's Rep protein lends an explanation to the RCR mechanism for BFDV genome replication. Mankertz *et al.* (1998) identified the Rep protein of PCV demonstrating the presence of three motifs involved in RCR and a putative dNTP-binding box (GKS) or P-loop that have also been identified in BFDV's Rep protein.

In geminiviruses, the conserved nonanucleotide motif is cleaved between the T and A at positions 7 and 8 of the motif (Bassami *et al.*, 1998; Todd, 2000; Maramorosch *et al.*, 2001). A tyrosine (Y) residue gives the Rep protein this nicking and binding function and was found to be conserved in all isolates by Ypelaar *et al.* (1999). Two repeats of an eight base pair (bp) sequence (GGGGCACC) adjacent to the potential stem-loop of BFDV may provide binding sites for Rep proteins prior to initiating RCR.

1.3.6: VIRUS PROTEINS AND ANTIGENS.

Purified preparations of BFDV reveal three structural proteins of 26.3 kDa, 23.7 kDa and 15.9 kDa as well as proteins with a molecular weight of 60 kDa. A study by Ritchie *et al.* (1990) revealed morphologically and antigenically similar isolates of BFDV and found that the major viral proteins from the isolates were similar. Minor protein bands in the 48 kDa and 58 kDa molecular weight range were observed and when the molecular weights of the smaller proteins (26.3, 23.7 and 15.9 kDa) were summed, they approximated a total weight of 60 kDa. It was speculated that these larger proteins could represent alternatively translated products or be host cell proteins which become viral associated during maturation. Additional structural proteins may be derived by proteolytic cleavage from the 26 kDa protein but this possibility must be further investigated.

Antigenically, BFDV is indicated to be similar worldwide. This was demonstrated by the ability to induce PBFD in an Umbrella cockatoo and African grey chick using virus purified from another Umbrella cockatoo. Furthermore, an Umbrella cockatoo and African grey parrot hen produced chicks that remained normal after virus challenge, after inoculation with BPL-treated BFDV recovered from a Moluccan cockatoo (Ritchie *et al.*, 1992a).

1.4: DISEASE ASPECTS.

1.4.1: EPIZOOTIOLOGY.

PBFD has been reported in free-ranging populations of psittacine birds as well as captive birds. Reports have been documented in Australia, North and South America (Brazil [Soares *et al.*, 1998]), Africa, South Pacific (Todd, 2000; Maramorosch *et al.*, 2001), Japan (Sanada *et al.*, 1999), Thailand (Kiatipattanasakul-Banlunara *et al.*, 2002), The Netherlands (Eisenberg *et al.*, 2003) and Germany (Rahaus & Wolff, 2003).

The geographic distribution of BFDV and the plant nanoviruses (with which BFDV shares homology) as well as the highest reported incidence of disease in psittacine birds native to the South Pacific islands and Australia reveals the existence of a possible common ancestor in the South Pacific (Niagro *et al.*, 1998). The worldwide movement of birds to meet the demands of the pet market facilitated the spread of PBFD to other continents. Introduction of BFDV into free-ranging populations of the world's more endangered psittacine species is continually encouraged by the intercontinental movement of birds and evidence already exists in South Africa where endangered Cape parrot populations have been recently diagnosed with PBFD.

Serological evidence indicates that, with some species, the prevalence of infection is high and greater than that of disease with Old World Psittaciformes appearing to be most likely infected due to their high susceptibility. Captive birds like galahs and budgerigars are thought to have a lower disease incidence than Sulphur crested cockatoos with as many as 20% of wild Sulphur crested cockatoos having clinical signs of PBFD in Australia in any one year (Maramorosch *et al.*, 2001). Epizootiologic studies in an import station in the United States of America (USA) indicated 0.5% of the imported lesser Sulphur crested cockatoos, Umbrella cockatoos, Citron cockatoos and Moluccan cockatoos had PBFD, suggesting that these birds had been infected in their country of origin (Ritchie *et al.*, 1991b; Ritchie & Carter, 1995). A 41% to 94% seroprevalence of BFDV infection in flocks of different free-ranging psittacine birds indicates the widespread infection in wild populations within Australia.

1.4.2.1: Natural Infections.

PBFD syndrome occurs predominantly in captive young birds less than three years old but has also been described in wild birds (Pass & Perry, 1984). Both sexes are affected and may become apparent with the first generation of contour feathers. The usual clinical course is progressive over several months to a year or more and is irreversible.

(A)   (B)
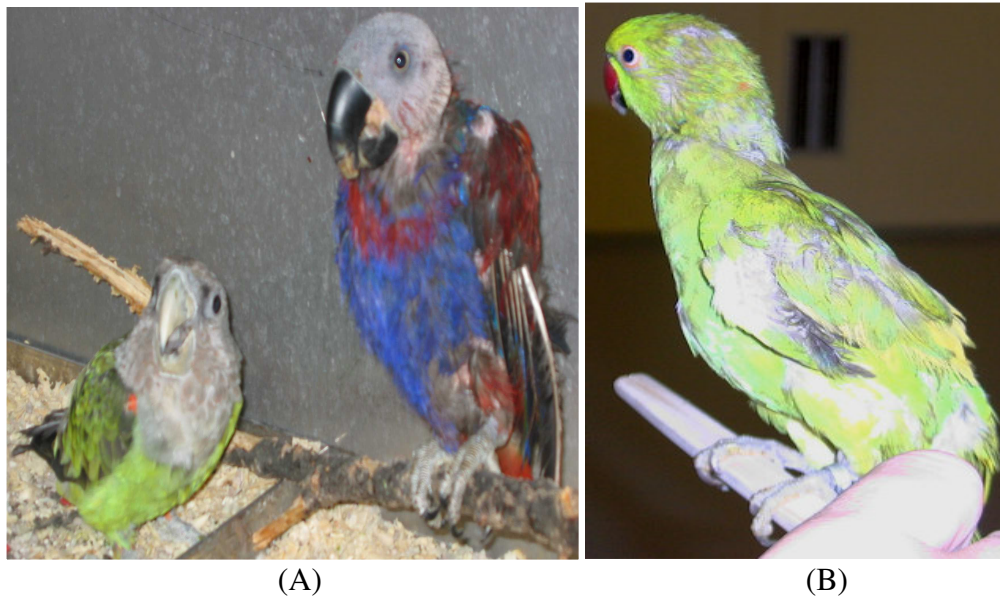
Figure 1.3: A Grey-headed parrot and Eclectus (A) showing signs of feather loss as well as a ring-neck parakeet (B). The Eclectus parrot is bald and suffers from beak deformities. The photographs were taken by Dr. J. Albertyn at the Animal House of the University of the Free State (UFS) where the birds are being cared for.

The first clinically detectable sign of PBFD is the appearance of necrotic, abnormally formed feathers with contour feathers and down being lost roughly symmetrically depending on the stage of molt when clinical signs are manifested (Figure 1.3). Normal plumage is replaced by abnormal feathers which may be short, clubbed, curled and deformed, may have retained sheaths, blood within

the shaft, circumferential constrictions and stress lines in the vane (Pass & Perry, 1984; Jergens *et al.*, 1988; Maramorosch *et al.*, 2001). Other symptoms include enteritis, septicemia, pneumonia, anaemia, depression and rapid weight loss.

In young birds (less than two months old), all of the feather tracts may be affected during a one-week period, whereas in older birds, the disease is more prolonged with progressive feather changes during ensuing molts (Ritchie & Carter, 1995). Some birds die shortly after the first indication of malformed feathers while others may live for several years in a featherless state [Figure 1.3] (Ritchie *et al.*, 1989).

The type of clinical disease (peracute, acute or chronic) varies markedly in clinical features and is controlled principally by the age of the bird when feather abnormalities first occur, but may also be influenced by the route of viral exposure, the titre of the infecting virus and the condition of the bird when viral exposure occurs (Ritchie & Carter, 1995).

A). Peracute infections should be suspected in neonatals exhibiting septicemia accompanied by pneumonia, enteritis, rapid weight loss and death. This type of infection is common in young cockatoos and African grey parrots where birds may die before feather abnormalities are recognized (Ritchie & Carter, 1995). A study by Schoemaker *et al.* (2000) on African grey parrots under seven months of age with PBFD revealed a consistent detection of severe leucopenia, anaemia or pancytopenia.

B). Acute infections are commonly reported in young birds during their first feather formation after the replacement of neonatal down. They are characterized by several days of depression followed by sudden changes in developing feathers (Ritchie & Carter, 1995). Diseased feathers may be shed prematurely, haemorrhage, fracture, bend or show signs of necrosis. In some cases, minimal feather changes may be accompanied by

depression, crop stasis and diarrhoea followed by death in one to two weeks.

C). Chronic disease symptoms include the symmetric, progressive appearance of abnormally developed feathers during each successive molt, retention of feather sheaths, haemorrhage within the pulp cavity, fractures of the feather shaft and abnormal feathers.

Powder down feathers are assumed to be affected first followed by the involvement of contour feathers and then the occurrence of dystrophic changes in the primary, secondary, tail and crest feathers. As the feather follicles become inactive, the surviving bird becomes bald (Figure 1.3).

Abnormal and uneven growth of the beak results in elongation, development of fault lines, breakage and under running of the outer and oral surface. The upper beak is more severely affected than the lower beak. Lesions in the beak give it a soft, greyish tone while in some birds the beak may appear to be semi-gloss or gloss black (Ritchie & Carter, 1995). Uneven wear, chips and bacterial infection contribute to impaired ability to eat and may lead to further debilitation and weight loss (Jergens *et al.*, 1988).

Beak pathology is not always present in birds with PBFD and seems dependent on the species involved and other unresolved factors (Ritchie *et al.*, 1989). Similar changes may occur in the claws which may eventually detach although it is a rare observance.

1.4.2.2: Experimental Infections.

BFDV was reproduced experimentally by inoculation of budgerigars and galahs with a homogenate prepared from affected feathers (Maramorosch *et al.*, 2001). Budgerigars inoculated prior to eight days of age developed more severe clinical

disease and lesions than those inoculated at 10 and 14 days, suggesting an age-related susceptibility to infection (Wylie & Pass, 1987). The main clinical abnormality was slow growth or lack of primary wing or tail feathers along with characteristic feather malformations and deformities (Maramorosch et al., 2001).

Experimentally infected Galah chicks developed clinical signs approximately four weeks after infection when they became depressed and anorectic. Thereafter, the feathers lost lustre becoming pale and brittle followed by feather dystrophy. Adult birds remain clinically normal and develop antibodies when experimentally infected with BFDV (Ritchie & Carter, 1995).

1.4.3: PATHOLOGICAL FEATURES.

1.4.3.1: Natural Infections.

Dystrophy and hyperplasia of the feather follicle, beak and claws result in the clinical abnormalities observed in PBFD. Necrosis and hyperplasia of the epidermal cells causes this dystrophy while hyperplasia produces hyperkeratosis of the feather sheath and outer layers of the beak and claws (Pass & Perry, 1984; Maramorosch et al., 2001). Widespread necrosis involves the entire pulp cavity characterized by suppurative inflammation involving the infiltration of heterophils, plasma cells, macrophages and lymphocytes.

Beak overgrowth is associated with hyperkeratosis and failure of the keratinized layers to slough (Jergens et al., 1988). Degenerative changes predispose the epidermal layers to splitting which can then become infected by bacteria. Atrophy of the thymus and bursa of Fabricius are observed in some cases suggesting an immunocompromised state of the affected bird.

Histologic evidence suggests that BFDV may be epitheliotropic in feathers and follicles, targeting replicating cells within the basal layer of the epithelium

(Latimer *et al.*, 1991). Basophilic intranuclear and intracytoplasmic viral inclusions were detected in epithelial cells whereas intracytoplasmic inclusions were often found in macrophages within the feather pulp cavity or feather epithelium. Infrequently, these inclusion-laden macrophages are present in the inner feather sheath, probably as a consequence of continued feather growth and repair despite viral infection (Latimer *et al.*, 1991).

Histologic changes associated with peracute PBFD may be limited to edema in the cells that line the feather follicle and severe necrosis of the bursa and thymus (Ritchie & Carter, 1995). Hepatic necrosis, secondary bacterial and fungal infections and lymphocellular depletion and atrophy in the bursa of Fabricius were found to characterize peracute BFDV infection in young African grey parrots under seven months old. In the acute form of PBFD, African grey parrots may die suddenly with histologic studies demonstrating virus-containing inclusion bodies in the bone marrow, thymus or bursa.

Trinkaus *et al.* (1998) identified BFDV infected cells with morphological alterations typical of apoptosis: condensation of cell cytoplasm, vacuole formation in the cytoplasm, nuclear chromatin condensation, zeiosis or outward blebbing (a phenomenon where vesicles bud from the cell surface appearing "to boil") and the appearance of apoptotic bodies (residues of degenerating cells) in the cytoplasm of dying and surrounding cells. The majority of affected cells were found in the intermediate layer of the feather follicle; a major region of keratin production, that when disrupted by cell death may be responsible for the deformity and loss of feathers.

1.4.3.2: Experimental Infections.

BFDV experimentally reproduced in budgerigars and galahs by inoculation with feather homogenates resulted in histologic lesions including necrosis of the cells lining the developing feather and the presence of large purple intracytoplasmic

inclusion bodies containing an accumulation of viral particles (Ritchie & Carter, 1995). Necrosis and depletion of lymphocytes in bursal follicles and thymic cortex have also been described (Maramorosch *et al.*, 2001).

1.4.4: PATHOGENESIS AND IMMUNOSUPPRESSION.

PBFD is a progressive disease with most affected birds surviving for less than six months to one year after the onset of clinical signs (Ritchie & Carter, 1995; Maramorosch *et al.*, 2001). However, some birds have been known to survive in a featherless state for over 10 to 15 years. An age-related window of susceptibility occurs and experimental infection studies have suggested a minimum incubation period for PBFD of 21 to 25 days. Juveniles aged between zero and three years affected by acute PBFD are thought to be susceptible due to host conditions rather than antigenic or genotypic traits of BFDV (Ritchie *et al.*, 2003). The time variance in developing clinical signs may be attributed to differences in concentrations of maternally-derived antibodies, titre of virus or host responses to the virus (Ritchie *et al.*, 1992a). The progress of the condition and the occurrence of new lesions may be halted or delayed during periods of non-molting (Maramorosch *et al.*, 2001).

Infection may involve a primary enteric site and/or the bursa of Fabricius, followed by viraemia (Raidal *et al.*, 1993a). The ability of the bursa to take up particulate matter from the cloaca is suggested to be involved in the age-related susceptibility. High faecal HA titres in a study by Raidal *et al.* (1993a) provided strong evidence that enteric infection is an important component of pathogenesis. Ritchie *et al.* (1991a) suggested that the gastrointestinal tract might be a site of replication and excretion of BFDV and later work by Raidal *et al.* (1993b) concluded that it is not the primary site of replication but a target site for BFDV replication and excretion.

Acute PBFD is associated with fatal liver disease and BFDV has been found to replicate in the liver early in the disease process. The replication continues into the chronic stage but the exact role the liver plays in the pathogenesis of BFDV must be further investigated.

The mechanism of viral infection of macrophages is unclear as macrophages may be primarily infected or may become infected during phagocytosis of virus-containing epithelial detritus (Latimer *et al.*, 1991). Inflammation is a frequent phenomenon in PBFD and BFDV is suggested to occur with a kind of cell death that morphologically resembles apoptosis (Trinkaus *et al.*, 1998).

Macrophages are critical for the initial processing and presentation of viral antigen to the immune system. Development of a chronic fatal BFDV infection or a protective immunologic response in an infected bird may be based on how the body processes the virus before it begins to persist in the cytoplasm of macrophages (Ritchie *et al.*, 1991b).

Neonatal psittacines infected mostly by peracute and acute PBFD exhibit a high incidence of mortality and severe pathologic changes in the bursa and thymus. An effective immune response will not be raised if they lack maternal antibodies against BFDV. Most adult PBFD infected birds develop transient viraemia then mount an effective immune response remaining asymptomatic.

Circoviruses damage lymphoid tissue, suppressing the immune system by targeting precursor T cells. This leads to the depletion of both helper (CD4^{+}) and cytotoxic (CD8^{+}) T cells (Ritchie *et al.*, 2003). Marked destruction of the bursa, persistence of the virus within the thymus and hypogammaglobulinemia indicating depressed antibody formation by B-lymphocytes in PBFD affected birds also indicate the immunocompromised state of the bird. Death of the bird results from secondary bacterial, chlamydial, fungal or other viral infections due to the immunosuppression. Trinkaus *et al.* (1998) also associated the necrosis

described in PBFD with these secondary infections and not with the virus-induced mechanism of pathogenicity.

1.4.5: EPIDEMIOLOGY.

Horizontal transmission through direct contact or through viral contaminated water or feeding areas is accelerated by the flocking nature of many birds susceptible to PBFD as the virus is highly contagious. Inhalation and ingestion of viral particles also serves as a transmission mode especially during preening and feeding activities. Although Ritchie *et al.* (1991a) found low concentrations of BFDV in the crop of positive birds, transmission of the virus to neonates during feeding which includes regurgitation of food and exfoliated crop epithelium should not be excluded. The source of virus could be attributed to infected cells in the crop or oesophageal epithelium or swallowed deposits of exfoliated epithelium from beak or oral mucosal lesions.

The high concentration of virus found in feather dust and the ease with which it can be dispersed both through natural air flow and through contact with clothing, nets, bird carriers, food dishes and insects indicates feather dust to be a major vehicle of transmission and environmental persistence of BFDV (Ritchie & Carter, 1995).

Recovery of BFDV from faeces suggests another mode of transmission. Nestling psittacine birds sit tripod-like on their legs and abdomen until they have a sense of balance. When they defaecate they rub their cloaca over nesting material allowing BFDV to gain access to the bursa of Fabricius by direct cloacal infection (Raidal *et al.*, 1993b). Psittacine chicks have been experimentally infected with BFDV via the oral, intramuscular, intranasal, intracloacal and subcutaneous routes.

Artificially incubated chicks from PBFD-infected hens consistently develop PBFD indicating vertical transmission of the virus from hen to eggs. Several reports indicate that asymptomatically infected adult birds can produce clinically infected progeny in successive breeding seasons (Maramorosch *et al.*, 2001). This suggests the existence of a carrier state from which horizontal or vertical transmission of BFDV may occur.

1.4.6: DIAGNOSIS.

Routine diagnosis of BFDV infection and the development of diagnostic tests and vaccines are restrained by the inability to propagate the virus in tissue or cell culture or in embryonated chicken eggs (Johne *et al.*, 2004). PBFD can be diagnosed by the use of histopathology to detect basophilic intranuclear and intracytoplasmic inclusion bodies but a confirmatory diagnosis requires the use of viral-specific antibodies to detect antigen or the detection of viral DNA due to the induction of similar-appearing inclusion bodies by other viruses. Other techniques that can be used for investigating PBFD are immunohistochemistry, transmission electron microscopy (TEM), agar-gel diffusion tests, the use of DNA probes and *in situ* hybridization and the enzyme-linked immunosorbent assay (ELISA).

Immunohistochemical staining with rabbit anti-BFDV antibodies has been used to confirm inclusion bodies in hematoxylin and eosin (H&E) stained tissue sections that contain BFDV antigen (Ritchie *et al.*, 1992b). The agar-gel diffusion test is based on the use of virus recovered from infected birds but is not very sensitive.

Viral-specific DNA probes detect BFDV nucleic acid in white blood cells in infected birds (Ritchie *et al.*, 1992a). Latimer *et al.* (1993) used BFDV and APV specific DNA probes to rapidly and economically confirm or exclude concurrent BFDV and APV infections in birds. *In situ* hybridization is less sensitive than DNA probes but together they form a better diagnostic tool. These probes can be used to biopsy samples of suspect feathers to confirm an infection or on a blood

sample to demonstrate viral nucleic acid before clinical changes in the feathers are apparent (Ritchie & Carter, 1995).

Johne *et al.* (2004) cloned part of the region encoding the capsid protein C1 and applied a polyhistidine-tailed variant of this protein as a recombinant antigen to test for BFDV-specific antibodies by an indirect ELISA and immunoblotting. Although individual BFDV isolates differ significantly within the C1 gene limiting the broad applicability of the use of such a recombinant antigen in serological tests, their results correlated well with the HI assays performed simultaneously. However, currently the most widely used serological test to detect BFDV antigen and antibodies is the HA and HI assays respectively because an optimized ELISA has not yet been commercially established.

The HA assay is currently the only method available for detecting BFDV that is also quantitative (Sanada & Sanada, 2000) while the HI is the first assay reported to determine and quantify BFDV-specific antibodies (Ritchie *et al.*, 1991b). HA assays can also be used for detecting routes of BFDV shedding from infected birds and the HI assay is a rapid test that can also be used to determine the seroprevalence of BFDV antibodies in captive and wild populations of psittacine birds (Ritchie *et al.*, 1991b).

Although the HA assay is useful, it is necessary to choose suitable erythrocytes because the HA activity of BFDV differs for erythrocytes of different species (Sanada & Sanada, 2000) though cockatoo erythrocytes have been described as being the most sensitive for detection. Ritchie *et al.* (1991b) and Sexton *et al.* (1994) reported on the occurrence of non-specific reactions in some serum samples due to HA and/or HA inhibitors that are not inactivated by heating and can result in false-positive reactions. Another drawback of using HA assays is that it does not detect incubating or latent BFDV infection. The possible genetic and antigenic diversity of BFDV further limits the applicability of this test resulting

in the need for standardized antigens to enable the comparison of tests (Johne *et al.*, 2004).

For the detection of nucleic acid, polymerase chain reaction (PCR) based techniques are commonly used. These techniques have been used to detect viral DNA in feather follicle material from clinically affected birds, in blood from asymptomatically infected birds and in swabbed material collected from cages and enclosures to ensure that environments are free from infection (Todd, 2000). PCR is probably the most sensitive for detecting latent or incubating BFDV infection but the results are not quantitative (Riddoch *et al.*, 1996).

A negative PCR result is a strong indication that a bird is not infected but a positive result should be interpreted in conjunction with the clinical signs, age of the bird and circulating antibody titres. Retesting after 90 days is recommended if a clinically normal appearing bird tested positive.

Ypelaar *et al.* (1999) developed a universal PCR test for the detection of BFDV based on the assumption that there is only one strain of BFDV worldwide. With the advent of technology, other forms of PCR tests have been performed: nested PCR (Kiatipattanasakul-Banlunara *et al.*, 2002) and real-time PCR (Raue *et al.*, 2004).

1.4.7: TREATMENT AND CONTROL.

Treatment of diseased birds is principally supportive and palliative at best (Jergens *et al.*, 1988). Due to extensive feather loss, thermoregulation in the affected birds is impaired and so they should be housed in warm, draught-free environments. Balanced diets must be provided along with antibiotic and other medication to combat secondary bacterial, fungal or parasitic infections. Antiviral drugs, immune system stimulants and herbal extracts may help to improve the

attitude or feather condition of an affected bird but they do not resolve the infection.

Although the cost of maintaining individual psittacine birds can be quite high, on a global or national scale the economic losses caused by BFDV infections are minimal. However, control strategies for PBFD do exist and can be very effective. Neonates which are most susceptible to PBFD must not be exposed to areas contaminated by faeces or feather dust from PBFD-positive birds. Equipment, caging and other facilities must be repeatedly and thoroughly cleansed to remove any residual virus shed by positive birds. Separate air flow systems in examination and treatment areas for PBFD-positive birds will prevent the spread of the virus by air. Asymptomatic birds can be sources of infection and should be PCR-tested to detect viral DNA.

Whether a bird is resistant to a virus or is fatally infected could depend on the age of the bird at the time of infection, the presence and concentrations of maternal antibodies, the route of viral exposure and/or the titre of the infecting virus (Ritchie *et al.*, 1992a). The most practical form of control would be the use of a vaccination programme whose success is guided by a number of factors: the ability of naturally exposed psittacines to remain clinically normal and develop a protective immune response, the indication that an antigenically similar virus infects a wide range of susceptible birds and experimental results indicating the protection of birds when vaccinated as well as the temporary protection chicks gain from vaccinated hens. The genetic variation between isolates from different psittacines lends an important view in the production of a vaccine. If they represent significant differences in antigenicity, pathogenicity or other physiochemical characteristics, a vaccine that would effectively protect a bird against any isolate(s) that may infect it at a particular time will need to be developed (Albertyn *et al.*, 2004).

Ritchie *et al.* (1992a) used viral preparations inactivated by BPL to inoculate a number of psittacine birds and thereby induced seroconversion in them. Their work also included the vaccination of Umbrella cockatoo and African grey parrot hens that produced chicks that were found to be temporarily resistant to BFDV when challenged suggesting that maternally transmitted antibodies can protect neonates from virus challenge. Raidal & Cross (1994) evaluated the use of a double-oil emulsion adjuvant vaccine (DOE-vacc) in a flock of *Agapornis spp.* where their results indicated that although it would not be able to eradicate BFDV from a flock, use of the vaccine with other biosecurity measures would be a safe and effective aid for controlling PBFD.

Additionally, effective legislative measures need to be implemented to control the sales of young birds incapable of flying or feeding themselves as this is when they are most susceptible to BFDV infection. Stricter law enforcement should be applied to curb the illegal trade market for these birds that is not only conducted across international but also intercontinental borders.

1.4.8: FUTURE WORK.

The inability to propagate BFDV in cell culture has limited the amount of investigation into PBFD and one of the most pertinent facets will be to try and find a cell culture system in which to cultivate the virus. Little is also known about the pathogenesis of BFDV, its replication and genetic diversity. What is the role of the liver in pathogenesis, what are the significances of genetic variation on antigenicity or pathogenicity, what level and type of antibodies are needed to protect a bird from infection and what is the critical period when these antibodies provide protection? These are but a few of the questions that are to date left insufficiently answered. Therefore, these and may more aspects of PBFD need to be investigated further if a better understanding of the disease is to be acquired.

1.5: CONCLUSION.

PBFD is a fatal disease that is increasingly becoming a worldwide problem. Although the economic losses caused by BFDV are not extreme on a national scale, individual losses incurred are leaving South African and bird breeders worldwide distraught. In addition, the aesthetic value of these birds and the survival of psittacine species, particularly endangered species like the Cape parrot, are being threatened by the mounting incidence of PBFD. The size of BFDV (being one of the smallest animal viruses) as well as the inability to propagate it *in vitro* makes the understanding of PBFD progression and the development of safe and effective control measures challenging. The search for a culture system for the growth of BFDV might prove lengthy but in the meantime the application of DNA technology will find use in the diagnosis and possibly development of a vaccine for the control of PBFD.

CHAPTER 2.

INTRODUCTION INTO THE PRESENT STUDY.

Psittacine beak and feather disease (PBFD) is the most common viral disease of wild Psittacine birds in Australia where it has endangered at least one species with extinction, but it is also a problem worldwide wherever captive psittacine birds are bred (Bassami *et al.*, 2001). Although it was first discovered in 1975 in Australia, PBFD was only very recently identified in psittacine birds in South Africa. The first documentation on the occurrence of PBFD in South Africa was by Albertyn *et al.* (2004) who identified PBFD in budgerigars and ring-neck parakeets.

In financial terms the bird breeder industry in South Africa may be larger than the cattle industry and continues to grow each year. One of the major interests in this industry lies in the maintenance and breeding of indigenous and exotic psittacine birds. Individual breeders invest substantial amounts of money in this market but recently have been incurring serious losses due to PBFD fatality. It has been suggested that the introduction of PBFD into the country may have occurred as a result of bird auctions and trade but most likely due to the illegal bird market that is transcending continental borders. Even more threatening than the loss of monetary investments is the extinction of the indigenous Cape parrot species (already on the endangered species list) that has indicated positivity for BFDV (beak and feather disease virus) infection.

A PCR (polymerase chain reaction) test is available commercially for the diagnosis of PBFD but currently there is minimal documentation on the study of the disease in South Africa. The greatest need is to develop a vaccine that can safely and effectively protect psittacine chicks from infection. Thus, extensive knowledge on BFDV infection in South African psittacines needs to be acquired for the development of such a vaccine. The initial step would then be to

investigate any genetic and/or antigenic diversity of isolates from South Africa with those currently identified in other countries to enable the production of a vaccine that can protect chicks from any isolate(s) [if a different strain is present in South Africa] that may infect it at a particular time. Presently it has been assumed that there is only one strain of BFDV worldwide but it is also likely that more than one strain exists like the two PCV (porcine circovirus) strains identified to date.

Hence, the aim of this study became to develop and optimize techniques that could be used to investigate both potential genetic and serologic differences in BFDV isolates obtained from different South African psittacines.

CHAPTER 3.

GENETIC ANALYSIS OF SOUTH AFRICAN BEAK AND FEATHER DISEASE VIRUS ISOLATES.

3.1: INTRODUCTION.

Studies have been performed on the genetic diversity of beak and feather disease (BFDV) in Australia (Bassami *et al.*, 2001; Raue *et al.*, 2004), New Zealand (Ritchie *et al.*, 2003) and southern Africa (Heath *et al.*, 2004). Based on the assumption that there is only one BFDV strain worldwide, Ypelaar *et al.* (1999) developed a universal polymerase chain reaction (PCR) for the detection of BFDV nucleic acid. This involved the amplification of a part (717 bp) of the open reading frame 1(ORF 1) which encodes the Rep protein, a highly conserved (87-99 %) region within the BFDV genome.

Although host factors are known to play a role in the differences in clinical and pathological manifestation of psittacine beak and feather disease (PBFD) in psittacines, the role of genetic variation in BFDV has been minimally investigated. Phylogenetic analysis of ORF 1 and ORF 2 by Bassami *et al.* (2001) revealed four clusters but there was little evidence to support a relationship between the regional distribution and genetic variation of the isolates.

Ritchie *et al.* (2003) showed a genotypic association with psittacine groups by analyzing sequences from ORF 1 of a variety of isolates and found three lineages present in New Zealand psittacines. They surmised that BFDV may be able to infect all psittacine species but that only certain genotypes are pathogenic in a particular species group. On the other hand, Raue *et al.* (2004) analyzed sequences of the ORF 2 from different isolates and even though they did not observe four groups (Bassami *et al.*, 2001), or the sequence diversity among

individual bird species (Ritchie *et al.*, 2003), their results indicate the possible existence of BFDV genotypes (especially in Lorikeets and African grey parrots).

A study of the coat protein of southern African BFDV isolates by Heath *et al.* (2004) revealed genetic diversity among them which led to the identification of three unique genotypes and eight lineages. In South Africa, Albertyn *et al.* (2004) investigated BFDV in Ring-neck parakeets and Budgerigars by performing restriction length fragment polymorphism (RFLP) studies. They found variations between the two species as well as between individuals from a single species. To further investigate the genetic variations of South African BFDV isolates, a phylogenetic analysis must be performed with these isolates and compared to isolates from other parts of the world.

The aim of this part of the research was the study of the occurrence of PBFD in South African psittacines. This was performed through the amplification of a region of the ORF 1 using PCR, and the determination if any genetic diversity exists between the BFDV isolates studied.

3.2: MATERIALS AND METHODS.

All reagents used were chemically pure unless otherwise stated and their suppliers are listed in Appendix A. Appendix B defines all buffer compositions used in the study.

3.2.1: COLLECTION OF BLOOD SAMPLES.

Blood samples were either collected from birds from a variety of psittacine species housed at the University of the Free State (UFS), subject to approval of the animal ethics committee Animal Project Number 15/02, that were tested over a period of two years or submitted on specimen collection paper by a number of breeders from different provinces in South Africa. Samples were obtained from

birds suspected to be PBFD positive with or without clinical signs. The specimen paper was prepared by radiating strips (5 cm by 0.5 cm) of Whatman number 1 filter paper in a GS GENE LINKER™ UV CHAMBER (BIORAD, cycle C4). Blood was collected (Figure 3.1) under the supervision of Dr. F. Potgieter, a qualified animal technician at the UFS Animal House, by pricking the wing vein of the bird (after disinfecting the concerned area) and spotting a drop of blood on the specimen paper to cover an area of approximately 5 mm by 5 mm. Samples were air-dried in a vertical position for at least 24 hours at room temperature (rt).

Figure 3.1: Blood sample collection from a Ring-neck parakeet showing clinical signs typical of PBFD. The bird is secured and the under side of the wing disinfected, the wing vein is pricked with a sterile needle and blood spotted onto specimen paper.

3.2.2: EXTRACTION OF DNA FROM DRIED BLOOD SAMPLES.

Viral DNA was extracted from the dried blood samples using the QIAamp DNA Mini Kit (QIAGEN) according to the Dried Blood Spot protocol in the manufacturer's instruction booklet.

The blood soaked area was cut into a clean 1.5 ml microcentrifuge tube and 180 µl of Buffer ATL added to it. The tube was incubated at 85 ^{o}C for 10 minutes (min) and briefly centrifuged (Biofuge 13, HERAEUS Instruments). Proteinase K

(20 µl) was added, the sample mixed by vortexing and incubated at 56 $^{\circ}$C for one hour. A volume of 200 µl of Buffer AL was added to the contents, mixed and the tube incubated at 70 $^{\circ}$C for 10 min after which it was centrifuged. A total of 200 µl of 99.5% ethanol was added to the sample, mixed and centrifuged. The sample was carefully applied to a QIAamp Spin Column (in a collection tube) and the column centrifuged at 6 000 g (8 000 revolutions per min [rpm]) for one min. Buffer AW1 (500 µl) was added to the column, centrifuged at 6 000 g for one min and the filtrate discarded. After placing the column in a clean collection tube, 500 µl of Buffer AW2 was added, the tube centrifuged at 20 000 g (13 000 rpm) for three min and the filtrate discarded. The column was placed back in the collection tube and centrifuged at 20 000 g for one min to eliminate buffer carryover. The column was then placed in a clean 1.5 ml microcentrifuge tube, 100 µl of Buffer AE added to it and incubated at rt for five min. The column was centrifuged at 6 000 g for one min and the eluted DNA stored at -20 $^{\circ}$C until required.

3.2.3: PCR AND RFLPs.

PCR was used to amplify part of the BFDV genome according to the method of Ypelaar *et al.* (1999) with slight modification. The primer pair (Table 3.1) PBF F1 and PBF R1 was used to amplify a 717 bp region within the ORF 1 of BFDV. Each reaction consisted of 5 µl of extracted viral DNA as template, 1 µl dNTPs (containing 10 mM of each dNTP), 0.5 µl of each primer (100 µM each), 5 µl of 10X concentration PCR buffer and 0.75 µl of SuperTherm DNA polymerase made up to a final volume of 50 µl with sterile Milli-Q water.

Table 3.1: Table indicating the primers used, their sequences, size and position in the BFDV genome for the amplification of part of the ORF 1.

PRIMER	SEQUENCE	SIZE (bp)	POSITION
PBF F1	5'-AACCCTACAGACGGCGAG-3'	18	182-199
PBF R1	5'- GTCACAGTCCTCCTTGTACC-3'	20	879-898

Reactions were thermocycled on Mastercycler Personal (Eppendorf®) using a hot start reaction where denaturation was carried out at 94 °C for 30 seconds (s) [initial step at 94 °C for 5 min after which the DNA polymerase was added to the reaction], annealing at 50 °C for 30 s and extension at 72 °C for 90 s for 30 cycles. PCRs of samples submitted from African grey parrots were annealed at 58 °C to reduce non-specific amplification.

Amplified products were identified by electrophoresis on 0.8% w/v agarose gels containing 0.01% w/v ethidium bromide. They were visualized under ultraviolet (U.V.) illumination together with λ phage DNA digested with *Eco*RI and *Hind*III as a molecular weight marker. Thereafter, all positive PCR products were digested at 37 °C for 3 hours using the restriction endonuclease enzyme *Hae*III. The total reaction (10 μl) consisted of 8.5 μl PCR product, 1 μl SuRE/cut buffer M and 0.5 μl enzyme *Hae*III. Restriction fragments were observed on 2% w/v agarose gels under U.V. illumination together with a 50 bp step ladder (Promega) as a molecular weight marker.

3.2.4: CLONING OF AMPLIFIED PRODUCTS FROM SIX ISOLATES (UFS 1-6) INTO pGEM™ TEasy VECTOR SYSTEM I.

To enable sequencing of the entire PCR product from both the 5' and 3' ends, amplicons from UFS 1, 2 and 6 were ligated into pGEM™ TEasy vector in a 10 μl reaction made up of 3.5 μl PCR or cloned product, 0.5 μl vector, 5 μl ligation buffer and 1 μl T4 DNA ligase. The reaction was incubated at 25 °C for at least 1 hour before being transformed into competent JM 109 *Escherichia coli* cells.

For each ligated sample, 200 μl of competent cells were defrosted on ice and 10 μl of the ligated plasmid added to it. For the negative control only competent cells were used. The mixture was placed on ice for 30 min, followed by 40 s at 42 °C and again on ice for 2 min. A volume of 800 μl of Luria Bertani [LB] broth was added to the sample and shaken at 37 °C for 1 hour. Samples were briefly

centrifuged and approximately 900 µl of the supernatant removed. The pellet was resuspended in the remaining 100 µl of supernatant and plated out on LB plates (supplemented with ampicillin, X-gal and IPTG). The plates were incubated overnight for 16 hours at 37 ^{o}C after which they were stored at 4 ^{o}C until required. Ten white colonies from each transformation of UFS 1, 2 and 6 were inoculated into 5 ml LB containing 50 µl (10 mg.ml^{-1}) ampicillin stock solution and grown overnight for 17 hours on a shaker at 37 ^{o}C.

Small-scale plasmid isolations were performed on samples UFS 1, 2 and 6 by alkaline lysis. Alkaline lysis was performed by filling a clean 1.5 ml microcentrifuge tube with the inoculated LB medium and the cells pelleted by centrifugation at 20 000 g for 1 min. The supernatant was aspirated and the pellet resuspended in 100 µl Glucose-Tris-EDTA [GTE] (2 M Glucose, 1 M Tris-HCl, 0.5 M EDTA) by vortexing. A volume of 200 µl of a NaOH/SDS (0.2 M NaOH, 1% w/v SDS) mixture was added, the tube inverted 5 times and placed on ice for 5 min. After the addition of 150 µl of potassium acetate [KAc] (5 M, pH 4.8) the tube was inverted 5 times and placed on ice for 5 min. The sample was centrifuged (Eppendorf Centrifuge 5417 R) at 20 000 g for 6 min at 4 ^{o}C and the supernatant was transferred to a clean microcentrifuge tube. Thereafter, 500 µl of isopropanol was added, mixed by vortexing and incubated at rt for 10 min. The tube was centrifuged at 20 000 g for 10 min at 4 ^{o}C, the supernatant aspirated and the pellet washed with 500 µl of ice-cold 70% ethanol by vortexing. This was followed by centrifugation at 20 000 g for 5 min at 4 ^{o}C after which the supernatant was aspirated and the pellet dried in a Speedvac Concentrator (SAVANT). The pellet was resuspended in 50 µl Tris-EDTA [TE] buffer (10 mM Tris, 1 mM EDTA, pH 8) containing 50 µg.ml^{-1} RNase and incubated at 37 ^{o}C for 30 min. The plasmid DNA was stored at -20 ^{o}C until required.

To confirm the insertion of the PCR product from each of the three isolates into the vector, restriction digests were performed on all the samples with *Eco*RI. Each digest sample of 3 µl of plasmid DNA, 0.5 µl *Eco*RI and 1 µl SuRE/cut

Buffer H was made up to a final volume of 10 µl with sterile Milli-Q water and incubated at 37 ^{o}C for 1 hour. The restriction fragments were resolved by electrophoresis on a 0.8% w/v agarose gel (0.01% w/v ethidium bromide) and observed under U.V. illumination with λ phage DNA digested with *Eco*RI and *Hind*III as a molecular weight marker.

Three additional samples (UFS 3, 4 and 5) were cloned into pGEM™ TEasy vector in a previous study (Albertyn *et al.*, 2004). These samples were retransformed in *E.coli* and purified using the GFX™ Microplasmid Prep Kit (Amersham Biosciences). A total volume of 1.5 ml of each inoculated LB medium was transferred to a clean 1.5 ml microcentrifuge tube and the cells pelleted by centrifugation at 20 000 *g* for 30 s. The supernatant was aspirated and the pellet resuspended in 150 µl of Solution I from the GFX™ Microplasmid Prep Kit by vigorous vortexing. A total volume of 150 µl of Solution II was then added and mixed by inverting the tube 10-15 times. After the addition of 300 µl of Solution III each sample was mixed by inverting the tube until a flocculent precipitate appeared and was evenly distributed (approximately 10-20 times). The tube was centrifuged at 20 000 *g* for 5 min to pellet cell debris and the supernatant was transferred to a GFX column in a collection tube. Each sample was incubated at rt for 1 min and centrifuged at 20 000 *g* for 30 s. The filtrate was discarded, 300 µl of Solution III was added to the sample then centrifuged at 20 000 *g* for 30 s. After the addition of 400 µl of wash buffer the sample was centrifuged at 20 000 *g* for 1 min and the filtrate discarded. The GFX column was transferred to a clean microcentrifuge tube and 50 µl of Tris buffer (5 mM, pH 8) added directly to the top of the matrix. It was incubated at rt for 1 min and centrifuged at 20 000 *g* for 1 min to recover purified DNA. The plasmid DNA was stored at -20 ^{o}C until required.

One positive clone from sample UFS 1, 2 and 6 together with UFS 3, 4 and 5 representing the six RFLPs I-VI were prepared for sequencing.

3.2.5: SEQUENCING OF UFS 1-6.

Samples UFS 1-6 were amplified in a sequencing PCR using the universal primer set T7 and Sp6. The reaction composition included 2 µl of premix (Big Dye® Terminator V3.1 Cycle Sequencing Kit, Applied Biosystems), 1-2 µl of plasmid template, 2 µl of either T7 or Sp6 primer (3.2 ρmoles) and 1 µl of 10X concentration PCR buffer made up to a final volume of 10 µl. The reactions were thermocycled on the Mastercycler Personal (Eppendorf®) at 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min for 25 cycles and ending with a cooling step at 4 °C.

Post-reaction cleanup was performed using the Sigmaspin™ Post Reaction Purification Columns (Sigma®). The seal of a column was broken, the column placed in a collection tube and centrifuged at 750 g for 2 min. The collection tube was discarded and the column placed in a clean 1.5 ml microcentrifuge tube. The sequencing sample was added to the centre of the column and centrifuged at 750 g for 4 min. The column was discarded and the sample dried completely in a Speedvac Concentrator (SAVANT). Sequencing was performed on an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems).

3.2.6: PHYLOGENETIC ANALYSIS OF SEQUENCES UFS 1-6.

The sequence data was assembled using AutoAssembler and reverse complimented and aligned using DNAssist V2.2 (http://www.dnassist.org/dnassist.htm). Analyzed sequences were compared to known BFDV Rep nucleotide sequences in the GenBank Database using a nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov), a tool made available by the NCBI to search nucleotide and protein databases for regions of similarity. The sequences were also translated into protein sequences and aligned using DNAMan (http://www.lynnon.com/).

Table 3.2: Beak and feather disease virus reference sequences used in this study.

Host species	Common name	Isolate	Origin	GenBank Accession no.
Psephotus haematogaster	Bluebonnet	BB-WA	Australia	AF311295
Agapornis roseicollis	Rosy-faced lovebird	LB-WA	Australia	AF311296
Cacatua tenuirostris	Eastern long-billed corella	ELBC-SA	Australia	AF311297
Eolophus roseicapillus	Galah	Galah-WA	Australia	AF311298
Trichoglossus haematodus	Rainbow lorikeet	LK-VIC	Australia	AF311299
Cacatua leabeateri	Major Mitchell's cockatoo	MMC-WA	Australia	AF311300
Cacatua galerita	Sulphur-crested cockatoo	SCC-NT	Australia	AF311301
C.galerita	Sulphur-crested cockatoo	SCC1-WA	Australia	AF311302
C.galerita	Sulphur-crested cockatoo	BFDV-AUS	Australia	AF080560
C.galerita	Sulphur-crested cockatoo	SCC2-NZ	New Zealand	AY148286
C.galerita	Sulphur-crested cockatoo	SCC3-NZ	New Zealand	AY148287
C.tenirostitus	Longbill corella	LC1-NZ	New Zealand	AY148289
C.galerita	Sulphur-crested cockatoo	SCC5-NZ	New Zealand	AY148290
Lorius chlorocercus	Yellow-bib lorikeet	YBL1-NZ	New Zealand	AY148292
T.haematodus	Rainbow lorikeet	RL5-NZ	New Zealand	AY148293
T.haematodus	Rainbow lorikeet	RL2-NZ	New Zealand	AY148294
Eos reticulata	Blue-streak lorikeet	BSL1-NZ	New Zealand	AY148296
E.reticulata	Blue-streak lorikeet	BSL2-NZ	New Zealand	AY148297
Psitteuteles goldei	Goldie's lorikeet	GL-NZ	New Zealand	AY148298
T.haematodus	Rainbow lorikeet	RL6-NZ	New Zealand	AY148300
Melopsittacus undulatus	Budgerigar	BG3-NZ	New Zealand	AY148301
Pionites leucogaster	White-bellied caique	WBC1-ZA	South Africa	AY450434
Psittacus erithacus	African grey parrot	AFG4-ZA	South Africa	AY450435
C.alba	White cockatoo	UC1-ZA	South Africa	AY450436
Poicephalus robustus	Cape parrot	CPA8-ZA	South Africa	AY450437
Poicephalus robustus	Cape parrot	CPA7-ZA	South Africa	AY450438
Poicephalus rueppellii	Rüppell's parrot	RP1-ZA	South Africa	AY450439
Poicephalus rufiventris	African red-bellied parrot	ARB4-ZA	South Africa	AY450440
Poicephalus gulielmi massaicus	Jardine parrot	GJP1-ZA	South Africa	AY450441
Agapornis nigrigensis	Black-cheeked lovebird	BCL1-ZAM	South Africa	AY450442
Psittacus erithacus	African grey parrot	AFG3-ZA	South Africa	AY450443

Sequences UFS 1-6 were phylogenetically analysed with the assistance of Livio Heath at the University of Cape Town. Reconstruction and bootstrapping of the

phylogenetic tree was performed in MEGA V2.1 (Kumar *et al.*, 2001). A neighbour-joining (N-J) tree, using sequences UFS 1-6 and a number ORF 1 sequences of BFDV isolates from different hosts obtained from GenBank(Table 3.2) which were edited in Bioedit and realigned, was constructed. The N-J tree was rooted with three non-psittacine avian circoviruses: goose circovirus (GenBank accession number AJ304456), canary circovirus (GenBank accession number AJ301633) and columbid circovirus (GenBank accession number AJ298229). For a test of phylogeny the tree was bootstrapped in MEGA in a 1000 trials using a random seed.

3.3: RESULTS AND DISCUSSION.

3.3.1: NUMBER AND DIVERSITY OF BLOOD SAMPLES TESTED.

Table 3.3: Table indicating number of birds tested for PBFD per each species and their results.

PSITTACINE SPECIES	ORIGIN	TOTAL NO. OF BIRDS TESTED	NO. OF POSITIVES	NO. OF NEGATIVES
Ringed neck parakeet	India	31	21	10
Blossom headed parakeet	India	1	-	1
Ruppel	Africa	3	2	1
Budgerigar	Australia	16	14	2
African grey parrot	Africa	22	4	18
Conure	South America	20	4	16
Senegal parrot	Africa	7	1	6
Amazon	South America	3	1	2
Eclectus	Australia	3	3	-
Jardine	Africa	25	15	10
Princess of Wales	Australia	1	-	1
Moustache	South East Asia	2	-	2
Stanley	South East Asia	2	1	1
Lorikeet	Australia	1	1	-
Red bellied parrot	Africa	1	1	-
Cape parrot	Africa	6	3	3
Lovebird	Africa	9	3	6
Brown headed parrot	Africa	7	-	7
Grey headed parrot	Africa	1	1	-
Total no. of birds		161	75	86

Blood sample collection on the specimen paper proved to be a rapid efficient method for specimen collection that did not require special transportation and storage facilities or tedious preparation for DNA extraction (Albertyn *et al.*, 2004).

This method of blood sample collection also enabled a large number of birds to be bled in a short period of time without causing any stress to them. The birds tested ranged from African to Australasian accumulating to a number of 19 different psittacine species (Table 3.3). Of the 161 samples tested, 75 birds [46.58%] tested positive for PBFD and 86 [53.42%] tested negative (Table 3.3).

Although the results obtained do not give a representation of the incidence of BFDV in South Africa as the birds tested were already suspected of suffering from PBFD, it may be possible that a similar percentage of birds that have not exhibited any clinical symptoms may be asymptomatic carriers of BFDV. This could have a fatal impact on bird breeders housing these asymptomatic birds since they are reservoirs of the virus and if they were to experience some stress, virus shedding could commence thereby exposing other birds to the virus. Consequently, it is strongly recommended that all birds in an established aviary be tested for PBFD if they had not been previously tested as well as any birds being introduced into the aviary.

3.3.2: PCR RESULTS FOR PSITTACINE BIRDS HOUSED AT THE UFS.

A number of different psittacine species suspected to be PBFD positive, donated from different sources for the purpose of the current study, were housed at the UFS and tested for BFDV infection by PCR over a period of one to two years depending on the time of their arrival at the UFS. The results of the PCR test varied with some of the birds being positive or negative throughout the testing schedule and others positive at a certain point and then seroconverting to negative (Table 3.4).

Some of the African grey parrots (numbers 962, 967, 968 and 970) and the Jardine number JUP11 that did not show clinical signs of PBFD all tested positive once during the testing period and a retest after a period of time yielded negative results. This could mean that the birds may have developed a subclinical

infection and mounted an immune response to it resulting in the production of antibodies to BFDV that cleared the infection hence, the seroconversion observed.

Table 3.4: Table indicating PCR results for PBFD tests carried out between January 2003 and May 2004 for psittacines housed at the UFS.

SPECIES	NO.	JAN 2003	FEB 2003	MARCH 2003	APRIL 2003	JULY 2003	SEPT 2003	NOV 2003	MAY 2004
					MONTH				
African grey parrot	943	-	-	+	N/A[1]	N/A		N/A	N/A
	956	-	-	-	-	-		-	-
	957	-	-	-	-	-		-	-
	959	-	-	-	-	-		-	-
	962	-	-	-	-	-		+	-
	965	+	+	+	+	+		+	+
	967	-	-	-	+	-		-	-
	968	-	-	-	+	-		-	-
	969	+	+	-	+	-		+	-
	970	-	-	+	+	-		-	-
Ringed neck parakeet	PB1	+						N/A	N/A
	PB2	+						N/A	N/A
	PB3	+						N/A	N/A
	PB4	+						N/A	N/A
	PB5	+						N/A	N/A
	PB6	+						N/A	N/A
	PBC17		-					N/A	N/A
	KB6		+					N/A	N/A
	KB8		+					+	+
	B2		-					+	N/A
	C1		-					+	N/A
	C3		-					+	N/A
Eclectus parrot	EB1		+					+	N/A
	EKH1					+		+	N/A
Cape parrot	CaPKW1					+		+	
	CaPUP1						+	+	
Lovebird	LBR1			+				N/A	N/A
	LBR2			+				N/A	N/A
	LBR3			+				+	+
	LBR4			-				N/A	N/A
Jardine	JUP8						-	-	
	JUP9						-	-	
	JUP10						-	-	
	JUP11						+	-	
	JUP12						-	-	
	JUP13						-	-	
	JUP14						-	-	

[1] N/A indicates a deceased bird.

Birds that had tested positive eventually died although whether they died from PBFD or secondary infections was unknown. Other positive birds especially African grey parrot number 965 and the Ringed neck parakeet number KB8 have survived in a featherless state from the time they were received.

However, an unusual set of results was obtained for African grey parrot 969 that did not exhibit any clinical signs of PBFD (Table 3.4). This bird tested positive for PBFD in January and February 2003, negative in March 2003 and a re-test in April 2003 indicated a positive result again. This phenomenon has continued in the testing schedule till May 2004 when the last test yielded a negative result. It may be possible that the virus was not present in the blood during the time of testing when results were negative but was residing in the organs. A study by Ritchie *et al.* (2003) that involved testing both feather and dried blood samples by PCR found that some samples that were obtained from the same bird would yield a positive result for the feather sample and not the dried blood sample and vice versa. The reliability of the PCR test as a diagnostic test might be questionable when blood samples are taken from asymptomatic birds if this phenomenon has been observed not once but twice already. This stresses the need for the establishment of a serological diagnostic test in South Africa that would be able to rapidly, efficiently and economically detect BFDV infections.

The results also indicate that most of the birds that may have developed subclinical infections as mentioned previously had initially tested negative. Birds like the African grey parrot 965 that have survived in a featherless state could be the source of infection as they are continuously shedding virus in feather dust and faeces that are well known transmission vehicles for BFDV. Even though the disinfectant Virukill Avisafe (MICROBROTICS), that is known to have strong disinfecting qualities against BFDV, was used regularly to wash the cages and feeding utensils, BFDV is an environmentally highly stable and infectious virus that is not eliminated easily from surfaces and so could have easily been transmitted to the negative birds in such a closed environment.

3.3.3: PCR AND RFLPs.

Positive PCR results were obtained for a number of birds from different psittacine species with an amplicon size of approximately 700 bp (Figure 3.2). In a study conducted previously (Albertyn *et al.*, 2004), digestion of the PCR products with *Hae*III yielded five different RFLPs [RFLP I-V]. In this study one additional RFLP profile was identified [RFLP VI] (Figure 3.3).
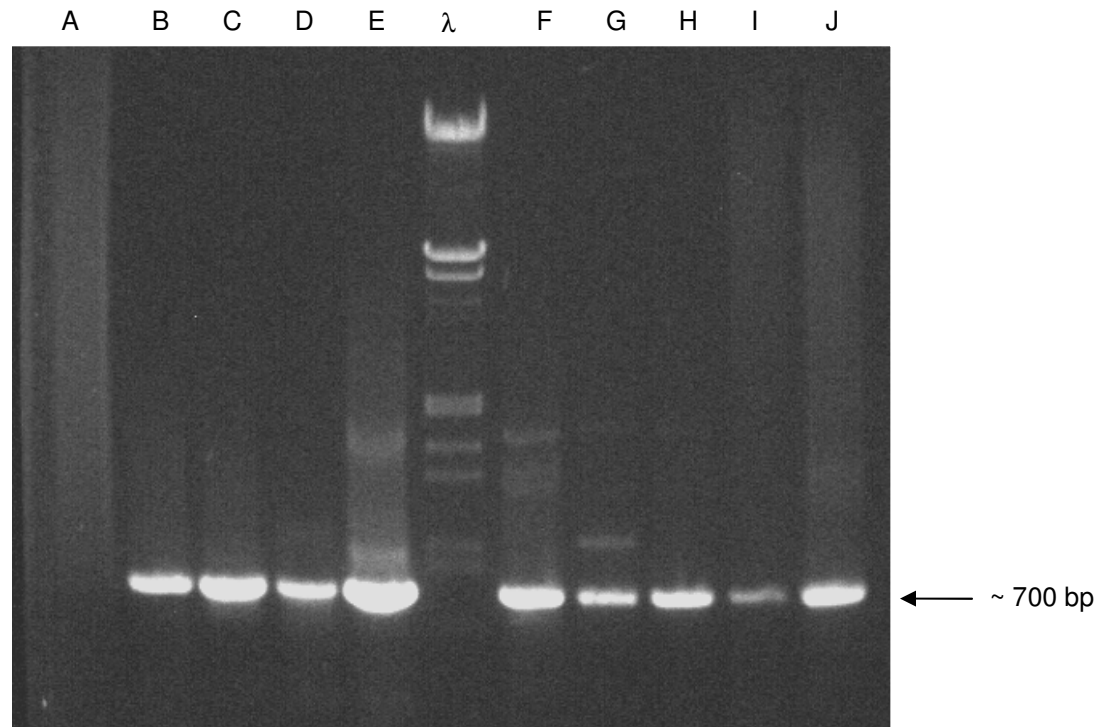
Figure 3.2: A representative gel photograph of PCR products for different psittacine species tested for PBFD. The PCR amplicon is approximately 700 bp in size. Lanes A and J represent the negative and positive controls used, respectively. Lanes B to I represent samples obtained from an African grey parrot, Ring-neck parakeet, Eclectus parrot, Cape parrot, Lovebird, Conure, Jardine and Budgerigar respectively. The samples were electrophoresed on a 0.8% w/v agarose gel and visualized under U.V. illumination together with a molecular weight marker (Lane λ) [λ *Eco*R1/*Hind*III].

RFLP I consisted of fragments approximately 450 bp, 250 bp and ~ 50 bp in size. RFLP II was similar with the two smaller fragments being the same size as RFLP

I but the largest fragment was approximately 460 bp. RFLP III, IV and V were only observed in samples obtained from Budgerigars that came from the Eastern Cape [RFLP IV and V] and Gauteng [RFLP III] provinces in South Africa (Table 3.5) and had three fragments each of approximate sizes 410 bp, 225 bp and 50 bp, 425 bp, 270 bp and 50 bp and 425 bp, 225 bp and 50 bp, respectively. RFLP VI was observed in blood samples obtained from a single farm in Kwa Zulu Natal province and consisted of fragments of approximately 425 bp, 250 bp, 50 bp and < 50 bp.
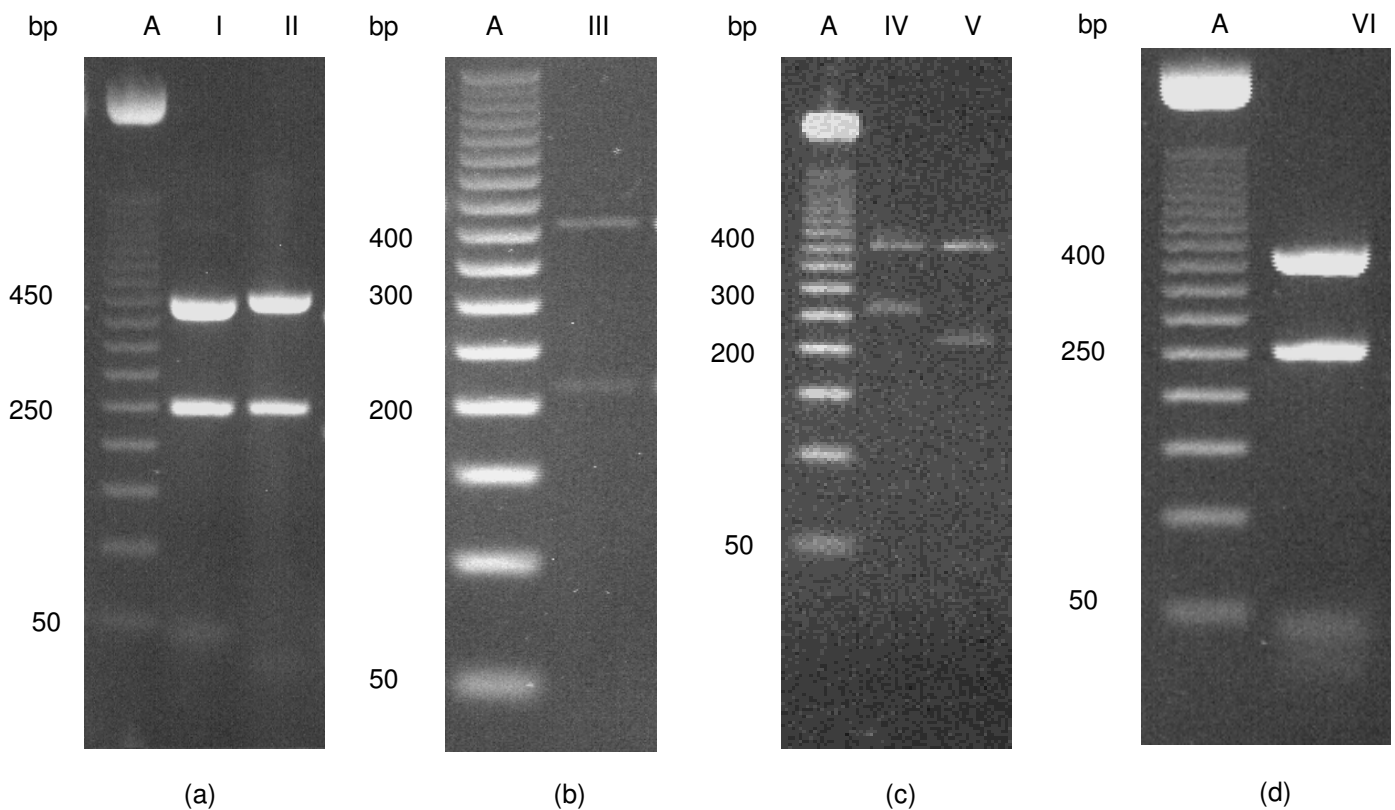
Figure 3.3: A representation of the five RFLPs I-V previously reported (Albertyn *et al.*, 2004) and RFLP VI that was observed in the present study. Restriction digests were electrophoresed on a 2% w/v low-melting agarose gel and observed under U.V. illumination together with a 50 bp DNA step ladder [Promega] (Lane A) as a molecular weight marker. The RFLPs I-VI were made up of fragments of approximately 450, 250 and ~ 50 bp, 460, 250 and ~ 50 bp, 410, 225 and 50 bp, 425, 270 and 50 bp, 425, 225 and 50 bp and 425, 250, 50 and < 50 bp, respectively.

The samples were obtained from six of the nine provinces in South Africa and from a diverse range of psittacine birds (Table 3.5). RFLP I was observed in all the species that tested positive for PBFD and was found in all the provinces. RFLP II was obtained from a single sample obtained from a Ringed neck parakeet and was the only sample submitted from the Western Cape province. It may be possible that if more samples were submitted from the Western Cape, there would have been a higher occurrence of RFLP II.

Table 3.5: Table indicating the psittacine species, source of sample and RFLP obtained.

SPECIES	SOURCE[*]	RFLP (bp)[†]
Ring neck parakeet	FS, WC	I, II
Budgerigar	EC, G, KZN, FS	I, III, IV, V
Ruppel	FS	I
African Grey parrot	FS, KZN	I
Senegal parrot	FS, G	I
Jardine parrot	G, KZN	I, VI
Red bellied parrot	KZN	I
Cape parrot	KZN	I, VI
Eclectus parrot	FS, KZN	I
Lorikeet	FS	I
Stanley	FS	I
Conure	FS, KZN	I, VI
Amazon	FS, G	I
Lovebird	LP, KZN	I
Grey headed parrot	FS	I

RFLPs III, IV and V were only observed in budgerigars and could be specific to this species although samples, obtained from budgerigars in the Free State

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[*] Provinces in South Africa where EC- Eastern Cape province, FS- Free State province, G- Gauteng province, KZN- Kwa Zulu Natal province, LP- Limpopo province and WC- Western Cape province.
[†] RFLP (bp) where: I- 450, 250 and ~ 50, II- 460, 250 and ~ 50, III- 410, 225 and 50, IV- 425, 270 and 50, V- 425, 225 and 50 and VI- 425, 250, 50 and < 50.

province, exhibited RFLP I. Although RFLP VI was observed in samples submitted from Jardines, Conures and a Cape parrot submitted from a single aviary in Kwa Zulu Natal, restriction digests of samples submitted from the same species type and the same province but from different aviaries yielded RFLP I (Table 3.5).

RFLP I appeared to be widespread in geographic distribution and infected a variety of psittacine species; in this study all the species that tested positive for PBFD. However, RFLP VI seemed to be confined to one aviary and a deeper enquiry into the history of the acquisition of these birds may lead to the identification of the primary source of this particular BFDV infection. There does not appear to be any regional or host adaptation where RFLP I is concerned. The same may be suggested for RFLP VI as the owner of these birds seems to have confined them and in so doing has prevented the spread of this infection to other provinces.

Although the RFLPs do not confirm any genetic diversity between the six profiles observed, it can be concluded that there is some genetic variation in the sequence of the ORF 1 from these isolates. RFLPs III, IV and V appear to be significantly different from the other RFLPs and could represent BFDV strains adapted to be pathogenic in Budgerigars only or possibly represent a novel circovirus in Budgerigars. These suggestions warranted further investigation into the six RFLPs which led to the sequencing of representative isolates to study any genetic diversity present.

3.3.4: PHYLOGENETIC ANALYSIS OF UFS 1-6.

The results of the nucleotide-nucleotide BLAST for all the sequences UFS 1-6 revealed more than 90% homology to isolates of BFDV from a variety of host species (Table 3.6).

Table 3.6: Table indicating nucleotide-nucleotide BLAST results for UFS 1-6 with isolates of highest percentage homology and their GenBank accession numbers.

SEQUENCE	HOST SPECIES /ISOLATE	ACCESSION NO	HOMOLOGY (%)
UFS 1	ARB4-ZA (*Poicephalus rufiventris*)	AY450440	96
	RP1-ZA (*Poicephalus rueppellii*)	AY450439	96
	CPA-7 (*Poicephalus robustus*)	AY450438	96
	CPA8-ZA (*Poicephalus robustus*)	AY450437	96
UFS 2	*Agapomis roseicollis*	AF311296	95
	Psephotus haematogaster	AF311295	95
	Cacatua leadbeateri	AF311300	95
UFS 3	*Cacatua galerita*	AF311302	95
	Eolophus roseicapillus	AF311298	95
	Cacatua leadbeateri	AF311300	95
UFS 4	*Cacatua galerita*	AF311302	96
	Cacatua leadbeateri	AF311300	96
	Eolophus roseicapillus	AF311298	96
UFS 5	*Cacatua leadbeateri*	AF311300	95
	Cacatua galerita	AF311302	95
UFS 6	*Cacatua leadbeateri*	AF311300	94
	AFG3-ZA (*Psittacus erithacus*)	AY450443	94
	Eolophus roseicapillus	AF311298	94
	Cacatua galerita	AF311302	94
	ARB4-ZA (*Poicephalus rufiventris*)	AY450440	94
	CPA8-ZA (*Poicephalus robustus*)	AY450437	94
	Cacatua tenuirostris	AF311297	94
	RP1-ZA (*Poicephalus rueppellii*)	AY450439	94
	CPA-7 (*Poicephalus robustus*)	AY450438	94

UFS 2, 3, 4 and 5 showed highest homology to previously described Australian BFDV isolates while UFS 6 showed highest homology to both Australian and southern African isolates described by Heath *et al.* (2004). UFS 1 indicated highest homology to some of the southern African isolates only. This is interesting because Heath *et al.* (2004) described three unique genotypes in southern Africa and it may be possible that UFS 1-6 may be included in these

genotypes. This would become clearer when interpreting the phylogenetic tree that was reconstructed.

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                                                                    →
UFS 1   1            NCNNNNNNTTGGCCGACTT-CAGCTCCGGCTGCA-GGCGGCCGCGGGAATTCGATTAACC  58
UFS 2   1          TGGNNNNNCGATGGCCGACTTTCAGCTCCGGCTCCATGGCGGCCGCGGGAATTCGATTAACC  62
UFS 3   1        GGCCCGATCCAAGCTCCCGGCGC-CAT------------GGCGGCCGCGGGAATTCGATTAACC  51
UFS 4   1  TGCGNNCNNNNNNNAATTGGCCGACTCTCNNAGTCGGTGTCC-GGAGGCCGCGGGAATTCGATTAACC  66
UFS 5   1                NTCCGGCTG-CCT------------GGCGGCCGCGGGAATTCGATTAACC  37
UFS 6   1          TTCTCAAG---GCGGCTGTCAT------------GGCGGCCGCGGGAATTCGATTAACC  44

UFS 1   59  CTACAGACGGCGAGATCGAATTCGTCCGTTGTCTGGGGCCTGACGAATTCTACTATGCCATCGTTGG  125
UFS 2   63  CTACAGACGGCGAGATCGAATTCGTGTGTTCGCTCGAGTCTGACGAATTCCACTATGCCATCGTTGG  129
UFS 3   52  CTACAGACGGCGAGATCGAATTCGTCCGTACTCTCGGGCCTGACGAATTCTTCTATGCCATCGTTGG  118
UFS 4   67  CTACAGACGGCGAGATCGAATTCGTCCGTACTCTCGGACCAGACGAATTCTACTATGCCATCGTTGG  133
UFS 5   38  CTACAGACGGCGAGATCGAATTCGTCCGTACTCTCGGGCCTGACGAATTCTACTATGCCATCGTTGG  104
UFS 6   45  CTACAGACGGCGAGATCGAATTCGTCCGTACTCTCGGGCCTGACGAATTCTACTATGCCATCGTTGG  111

UFS 1   126  ACGGGAAAAGGGCGAGCAAGGTACCCCCCATTTGCAAGGCTACTTTCATTTTAAAAATAAGAAGCGA  192
UFS 2   130  ACGGGAAAAAGGCGAGCAAGGTACCCCCCATTTGCAAGGCTACTTTCATTTTAAAAATAAGAAGCGA  196
UFS 3   119  ACGGGAAAAGGGCGAGCAAGGTACTCCCCATTTGCAAGGCTACTTTCATTTTAAAAATAAGAAGCGA  185
UFS 4   134  ACGGGAAAAGGGCGAGCAAGGTACCCCCCATTTGCAAGGCTACTTTCATTTTAAAAATAAGAAGCGA  200
UFS 5   105  ACGGGAAAAGGGCGAGCAAGGTACCCCCCATTTGCAAGGCTACTTTCATTTTAAAAATAAGAAGCGA  171
UFS 6   112  ACGGGAGAAGGGCGAGCAAGGTACCCCCCATTTGCAAGGCTACCTCCATTTTAAAAATAAGAAGCGA  178

UFS 1   193  CTGAGCGCGCTTAAGAAAATGCTGCCGCGAGCTCATTTTGAGCGCGCTAAAGGGAGCGATGCGGACA  259
UFS 2   197  CTGAGCGCGCTTAAGAAAATGCTGCCGCGAGCTCATTTTGAGCGCGCTAAAGGGAGCGATGCGGACA  263
UFS 3   186  CTGAGCGCGCTTAAGAAAATGCTGCCGCGAGCTCATTTTGAGCGCGCTAAAGGGAGTGATGCTGATA  252
UFS 4   201  CTGAGCGCGCTTAAGAAAATGCTGCCGCGAGCTCATTTTGAGCGCGCTAAAGGGAGCGATGCGGATA  267
UFS 5   172  CTGAGCGCGCTTAAGAAAATGCTGCCGCGAGCTCATTTTGAGCGCGCTAAAGGGAGTGATGCAGATA  238
UFS 6   179  CTGAGCGCGCTTAAGAAAATGCTGCCGCGAGCACATTTTGAGCGCGCTAAAGGGAGCGATGCGGAGA  245

UFS 1   260  ATGAGAAGTATTGCAGTAAGGAGGGGGACGTTATACTTACCCTGGGCATTGTGGCGAGAGACGGTCA  326
UFS 2   264  ATGAGAAGTATTGCAGCAAAGAGGGGGACGTGATACTTACCCTGGGCATTGTGGCGAGAGACGGTCA  330
UFS 3   253  ATGAGAAGTATTGCAGTAAAGAGGGGGACGTTATACTTACCCTGGGCATTGTGGCGAGAGATGGTCA  319
UFS 4   268  ATGAGAAGTATTGCAGTAAAGAGGGGGACGTTATACTTACCCTGGGCATTGTGGCGAGAGACGGTCA  334
UFS 5   239  ATGAGAAGTATTGCAGTAAAGAGGGGGACGTTATACTTACCCTGGGCATTGTGGCGAGAGACGGTCA  305
UFS 6   246  ATGAGAAGTATTGCAGTAAAGAGGGGGACGTAATACTTACCCTGGGCATTGTGGCGAGAGACGGTCA  312

UFS 1   327  CCGCGCTTTTGACGGAGCTGTTGCTGCCGTGATGTCCGGACGCAAAATGAAGGAAGTCGCGCGAGAG  393
UFS 2   331  CCGCGCTTTCGACGGAGCTGTTGCTGCCGTGATGTCCGGACGCAAAATGAAGGAAGTCGCGCGAGAG  397
UFS 3   320  CCGCGCTTTCGACGGAGCTGTTGCTGCCGTGATGTCCGGACGCAAAATGAAGGAAGTCGCGCGAGAG  386
UFS 4   335  CCGCGCTTTCGACGGAGCAGTTGCTGCCGTGATATCCGGACGCAAAATGAAGGAAGTCGCGCGAGAG  401
UFS 5   306  CCGCGCTTTCGACGGAGCTGTTGCTGCCGTGATGTCCGGACGCAAAATGAAGGAAGTCGCGCGAGAG  372
UFS 6   313  CCGCGCGTTCGATGGAGCTGTTGCTGCCGTGATGTCCGGACGCAAAATGAAGGAAGTCGCGCGAGAG  379

UFS 1   394  TTCCCAGATATCTACGTCAGGCATGGGCGGGGCTTGCATAGCCTCTCGCTATTGGTGGGTTCCCGCC  460
UFS 2   398  TTCCCAGATATCTACGTCAGGCATGGGCGGGGCTTGCATAACCTCTCGCTATTGGTTGGTTCCCGCC  464
UFS 3   387  TTCCCAGATATCTACGTCAGGCATGGGCGGGGCTTGCATAACCTCTCGCTATTGGTCGGTTCCCGCC  453
UFS 4   402  TTCCCAGATGTCTACGTCAGGCATGGGCGGGGCTTGCATAACCTCTCGCTATTGGTCGGTTCCCGCC  468
UFS 5   373  TTCCCTGATATCTACGTCAGGCATGGGCGGGGCTTGCATAACCTCTCGCTATTGGTCGGTTCCCGCC  439
UFS 6   380  TTCCCAGATATCTACGTCAGGCATGGGCGGGGCTTGCATAGTCTCTCGCTAATGGTCGGTTCCCGTC  446
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Figure 3.4: Alignment of sequences UFS 1-6 which form part of the ORF 1 of BFDV showing areas of high nucleotide sequence homology. The forward arrow indicates the beginning of the amplified region and the reverse arrow, the end of the amplified region.

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UFS 1  461  CATGTGACTTCAAGACCGAGGTTGACGTCATCTACGGACCACCGGGGTGTGGCAAGAGCAGATGGGC  527
UFS 2  465  CACGTGACTTCAAGACTGAGGTTGACGTCATCTACGGCCCACCGGGGTGTGGCAAGAGTAGATGGGC  531
UFS 3  454  CACGTGATTTCAAGACAGAAGTTGACGTCATCTACGGGCCCCCTGGGTGTGGCAAGAGTAAATGGGC  520
UFS 4  469  CACGTGATTTCAAGACAGAGGTTGACGTCATCTACGGGCCACCTGGGTGTGGCAAGAGTAAATGGGC  535
UFS 5  440  CACGTGATTTCAAGACAGAAGTTGACGTCATCTACGGGCCACCTGGGTGTGGCAAGAGTAAATGGGC  506
UFS 6  447  CACGTGATTTCAAGACTGAGGTTGACGTAATCTACGGGCCACCGGGGTGTGGCAAGAGTAGATGGGC  513

UFS 1  528  CAATGAGCAGCCTGGGACAAAATTTTATAAAATGCGCGGTGAATGGTGGGATGGATATGATGGGGAG  594
UFS 2  532  CAATGAGCAGCCTGGGACTAAATATTATAAAATGCGGGGTGAATGGTGGGATGGCTATGATGGGGAA  598
UFS 3  521  CAATGAGCAGCCTGGGACTAAATATTATAAAATGCGCGGTGAATGGTGGGATGGATATGATGGGGAA  587
UFS 4  536  CAATGAGCAGCCCGGGACTAAATATTATAAAATGCGCGGTGAATGGTGGGATGGATATGATAGGGAA  602
UFS 5  507  CAATGAACAGCCTGGGACTAAGTATTATAAAATGCGCGGTGAATGGTGGGATGGATATGATGGGGAA  573
UFS 6  514  CAATGAGCAGCCTGGGACTAAATATTATAAGATGCGCGGGGAATGGTGGGATGGATATGATGGGGAA  580

UFS 1  595  GAAGTCGTCATCTTGGACGACTTTTATGGGTGGCTACCTTATTGCGAGTTGCTCCGCCTCTGTGACC  661
UFS 2  599  GAAGTAGTCGTATTGGACGACTTCTATGGGTGGCTACCCTATTGCGAGATGCTCCGCCTCTGCGACC  665
UFS 3  588  GATGTTGTCATATTGGACGACTTTTATGGGTGGCTACCTTATTGTGAGATGCTCCGCCTTTGCGACC  654
UFS 4  603  GATGTCGTTATATTGGATGACTTTTATGGGTGGCTACCTTATTGCGAGATGCTCCGCCTCTGCGACC  669
UFS 5  574  GATGTCGTCATATTGGACGACTTTTATGGGTGGCTACCTTATTGTGAGATGCTCCGCCTCTGCGACC  640
UFS 6  581  GATGTCGTCATCTTGGACGACTTTTATGGGTGGCTACCTTATTGCGAGATGCTCCGCCTCTGTGACC  647

UFS 1  662  GTTACCCACATAAAGTGCCAGTTAAGGGCGCTTTTGTGGAGTCTACAAGCAAGAGGATCATTATCAC  728
UFS 2  666  GTTACCCACATAAGGTGCCAGTTAAGGGCGCCTTTGTGGAGTTTACGAGCAAGAGGATCATTATCAC  732
UFS 3  655  GTTATCCACATAAAGTGCCAGTTAAGGGCGCTTTTGTGGAGTTTACCAGCAAGAGGATCATTATCAC  721
UFS 4  670  GTTACCCACATAAAGTGCCAGTTAAGGGCGCTTTTGTGGAGTTTACCAGCAAGAGGATCATTATCAC  736
UFS 5  641  GTTACCCACATAAAGTGCCAGTTAAGGGCGCTTTTGTGGAGTTTACCAGTAAGAGGATCATTATCAC  707
UFS 6  648  GTTACCCACACAAAGTGCCAGTTAAGGGCGCTTTCGTGGAGTTTACCAGCAAGAGGATAATTATCAC  714

UFS 1  729  AAGCAATAAGCCCCCCGAGACCTGGTACAAGGAGGACTGTGACAATCACTAGTGAATTCGCGGCCGC  795
UFS 2  733  GAGCAATAAGTCCCCCGAGAACTGGTACAAGGAGGACTGTGACAATCACTAGTGAATTCGCGGCCGC  799
UFS 3  722  GAGCAATAAGTCCCCCGAGACCTGGTACAAGGAGGACTGTGACAATCACTAGTGAATTCGCGGCCGC  788
UFS 4  737  GAGCAATAAGGCCCCCGAGACTTGGTACAAGGAGGACTGTGACAATCACTAGTGAATTCGCGGCCGC  803
UFS 5  708  GAGCAATAAGGCCCCCGAGACCTGGTACAAGGAGGACTGTGACAATCACTAGTGAATTCGCGGCCGC  774
UFS 6  715  GAGCAATAAGTCCCCCGAGACCTGGTACAAGGAGGACTGTGACAATCACTAGTGAATTCGCGGCCGC  781

UFS 1  796  CTGCAGGTCGACCATATGGAGAGTCCC--ACGCTTG-GANNTAGTT-ANTNNNNNNNCNT  851
UFS 2  800  CTGCAGGTCGACCATATGGTTAGGACCCAACGCTTGAGAGCTAGCTGAGTNNNNNNGCNA  859
UFS 3  789  CTGCAGGTCGACCATATGGAGAGCACCCAAC-CTTGGA-GCTAGTG-AGTANGNGNNCA   844
UFS 4  804  CTGCAGGTCGACCATATGGAGAGCTCCCAACGCTTGGAAGCTA                  846
UFS 5  775  CTGCAGGTCGACCATATGGAGAGCACCCAACGCTTGGA-ANNN                  816
UFS 6  782  CTGCAGGTCGACCATATGGAGAGCACCCAAC--CTNGAAGCTAGTC-AATTNNNTGN    835
            ←
```

Figure 3.4 (Continued): Alignment of sequences UFS 1-6 which form part of the ORF 1 of BFDV showing areas of high nucleotide sequence homology. The forward arrow indicates the beginning of the amplified region and the reverse arrow, the end of the amplified region.

The nucleotide alignment performed with sequences UFS 1-6 showed many areas of homology with a few base differences (Figure 3.4). These results show the occurrence of a highly conserved nucleotide sequence for the Rep protein in accordance with previous reports.

```
UFS1  GGRGNSINPTDGEIEFVRSLGPDEFYYAIVGREKGEQGTP      40
UFS2  GGRGNSINPTDGEIEFVCSLESDEFHYAIVGREKGEQGTP      40
UFS3  GGRGNSINPTDGEIEFVRTLGPDEFFYAIVGREKGEQGTP      40
UFS4  GGRGNSINPTDGEIEFVRTLGPDEFYYAIVGREKGEQGTP      40
UFS5  GGRGNSINPTDGEIEFVRTLGPDEFYYAIVGREKGEQGTP      40
UFS6  GGRGNSINPTDGEIEFVRTLGPDEFYYAIVGREKGEQGTP      40
```

PYROPHOSPHATASE DOMAIN   RCR 1

```
UFS1  HLQGYFHFKNKKRLSALKKMLPRAHFERAKGSDADNEKYC      80
UFS2  HLQGYFHFKNKKRLSALKKMLPRAHFERAKGSDADNEKYC      80
UFS3  HLQGYFHFKNKKRLSALKKMLPRAHFERAKGSDADNEKYC      80
UFS4  HLQGYFHFKNKKRLSALKKMLPRAHFERAKGSDADNEKYC      80
UFS5  HLQGYFHFKNKKRLSALKKMLPRAHFERAKGSDADNEKYC      80
UFS6  HLQGYLHFKNKKRLSALKKMLPRAHFERAKGSDAENEKYC      80
```

RCR 1   RCR 2

```
UFS1  SKEGDVILTLGIVARDGHRAFDGAVAAVMSGRKMKEVARE     120
UFS2  SKEGDVILTLGIVARDGHRAFDGAVAAVMSGRKMKEVARE     120
UFS3  SKEGDVILTLGIVARDGHRAFDGAVAAVMSGRKMKEVARE     120
UFS4  SKEGDVILTLGIVARDGHRAFDGAVAAVISGRKMKEVARE     120
UFS5  SKEGDVILTLGIVARDGHRAFDGAVAAVMSGRKMKEVARE     120
UFS6  SKEGDVILTLGIVARDGHRAFDGAVAAVMSGRKMKEVARE     120
```

RCR 2

```
UFS1  FPDIYVRHGRGLHSLSLLVGSRPCDFKTEVDVIYGPPGCG     160
UFS2  FPDIYVRHGRGLHNLSLLVGSRPRDFKTEVDVIYGPPGCG     160
UFS3  FPDIYVRHGRGLHNLSLLVGSRPRDFKTEVDVIYGPPGCG     160
UFS4  FPDVYVRHGRGLHNLSLLVGSRPRDFKTEVDVIYGPPGCG     160
UFS5  FPDIYVRHGRGLHNLSLLVGSRPRDFKTEVDVIYGPPGCG     160
UFS6  FPDIYVRHGRGLHSLSLMVGSRPRDFKTEVDVIYGPPGCG     160
```

P-LOOP

```
UFS1  KSRWANEQPGTKFYKMRGEWWDGYDGEEVVILDDFYGWLP     200
UFS2  KSRWANEQPGTKYYKMRGEWWDGYDGEEVVVLDDFYGWLP     200
UFS3  KSKWANEQPGTKYYKMRGEWWDGYDGEDVVILDDFYGWLP     200
UFS4  KSKWANEQPGTKYYKMRGEWWDGYDREDVVILDDFYGWLP     200
UFS5  KSKWANEQPGTKYYKMRGEWWDGYDGEDVVILDDFYGWLP     200
UFS6  KSRWANEQPGTKYYKMRGEWWDGYDGEDVVILDDFYGWLP     200
```

P-LOOP

Figure 3.5: Protein sequence alignment of UFS 1-6 showing high percentage identity (98.05%). The sequences differ by a few amino acids although most of them belong to the same group of amino acids. Two of the conserved motifs for rolling circle replication are present (red underline). The P-loop sequence (blue underline) and the putative pyrophosphatase domain (broken blue line, underlined) are also shown.

```
UFS1    YCELLRLCDRYPHKVPVKGAFVESTSKRIIITSNKPPETW    240
UFS2    YCEMLRLCDRYPHKVPVKGAFVEFTSKRIIITSNKSPENW    240
UFS3    YCEMLRLCDRYPHKVPVKGAFVEFTSKRIIITSNKSPETW    240
UFS4    YCEMLRLCDRYPHKVPVKGAFVEFTSKRIIITSNKAPETW    240
UFS5    YCEMLRLCDRYPHKVPVKGAFVEFTSKRIIITSNKAPETW    240
UFS6    YCEMLRLCDRYPHKVPVKGAFVEFTSKRIIITSNKSPETW    240


UFS1    YKEDCDN    247
UFS2    YKEDCDN    247
UFS3    YKEDCDN    247
UFS4    YKEDCDN    247
UFS5    YKEDCDN    247
UFS6    YKEDCDN    247
```

Figure 3.5 (Continued): Figure 3.5: Protein sequence alignment of UFS 1-6 showing high percentage identity (98.05%). The sequences differ by a few amino acids although most of them belong to the same group of amino acids. Two of the conserved motifs for rolling circle replication are present (red underline). The P-loop sequence (blue underline) and the putative pyrophosphatase domain (broken blue line, underlined) are also shown.

The results from the BLAST and the alignment of the nucleotide sequences helped to eliminate the possibility that UFS 3, 4 and 5 (RFLPs III, IV and V) could represent a novel circovirus in Budgerigars. Whether or not these three isolates are pathogenic in budgerigars only will require more investigation.

Alignment of the protein sequences for UFS 1-6 (Figure 3.5) resulted in a 98.05% identity measure. The differences in protein sequence alignment as compared to nucleotide sequence alignment were lesser and reiterate the conservation observed in the gene encoding the Rep protein.

Two of the three conserved motifs for rolling circle replication (RCR) were present in the amplified sequence as well as the P-loop motif. The P-loop is said to be involved in nucleotide binding (Maramorosch et al., 2001) and is associated with dNTPase activity. The two motifs involved in RCR were identical in all the isolates except for one amino acid change in the first motif (RCR 1) of UFS 6. An important factor which determines the function of the replication-associated

proteins in circular ss-DNA replicons exploiting RCR, is the tyrosine residue [Y] (Ypelaar *et al.*, 1999). From the alignment in Figure 3.5 the conservation of the tyrosine residue in both the motifs (PHLQGYF and EKYCSK) can be observed for all six of the isolates. The tyrosine gives the Rep protein the nicking and binding function that initiates RCR thus, emphasizing the involvement of RCR in these BFDV isolates.

In order to have a more meaningful interpretation of the genetic diversity in isolates UFS 1-6 and those BFDV isolates whose sequences have been deposited in GenBank the use of a nucleotide phylogenetic tree (Figure 3.6) provided more information regarding genetic diversity than the RFLPs. A neighbour-joining (N-J) tree was constructed in MEGA V2.1 (Kumar *et al.*, 2001) and was rooted with three non-psittacine avian circoviruses: goose circovirus (GenBank accession number AJ304456), canary circovirus (GenBank accession number AJ301633 and columbid circovirus (GenBank accession number AJ298229). The topology of the tree remained the same each time the tree was rooted and a subtree of the BFDV isolates can be seen in Figure 3.6. The topology of the subtree resembles the maximum-likelihood tree depicting the phylogenetic relationships of Rep of BFDV in the study by Heath *et al.* (2004).

The three lineages, cockatoo (CK*), budgerigar (BG*) and lorikeet (LK*), described by Ritchie *et al.* (2003) were supported by significant bootstrap values (≥ 82%). A fourth lineage, lovebird (LB*), described by Heath *et al.* (2004) was also present. Individual Rep sequences of isolates UFS 1, 2 and 6 clustered separately from other isolates but UFS 3, 4 and 5, which were obtained from budgerigars, clustered together with the isolate BG3-NZ which represents the BG* lineage. These results reiterate the results obtained by Ritchie *et al.* (2003) who found a genotypic association with psittacine species. Thus, UFS 3, 4 and 5 can be placed in this lineage.
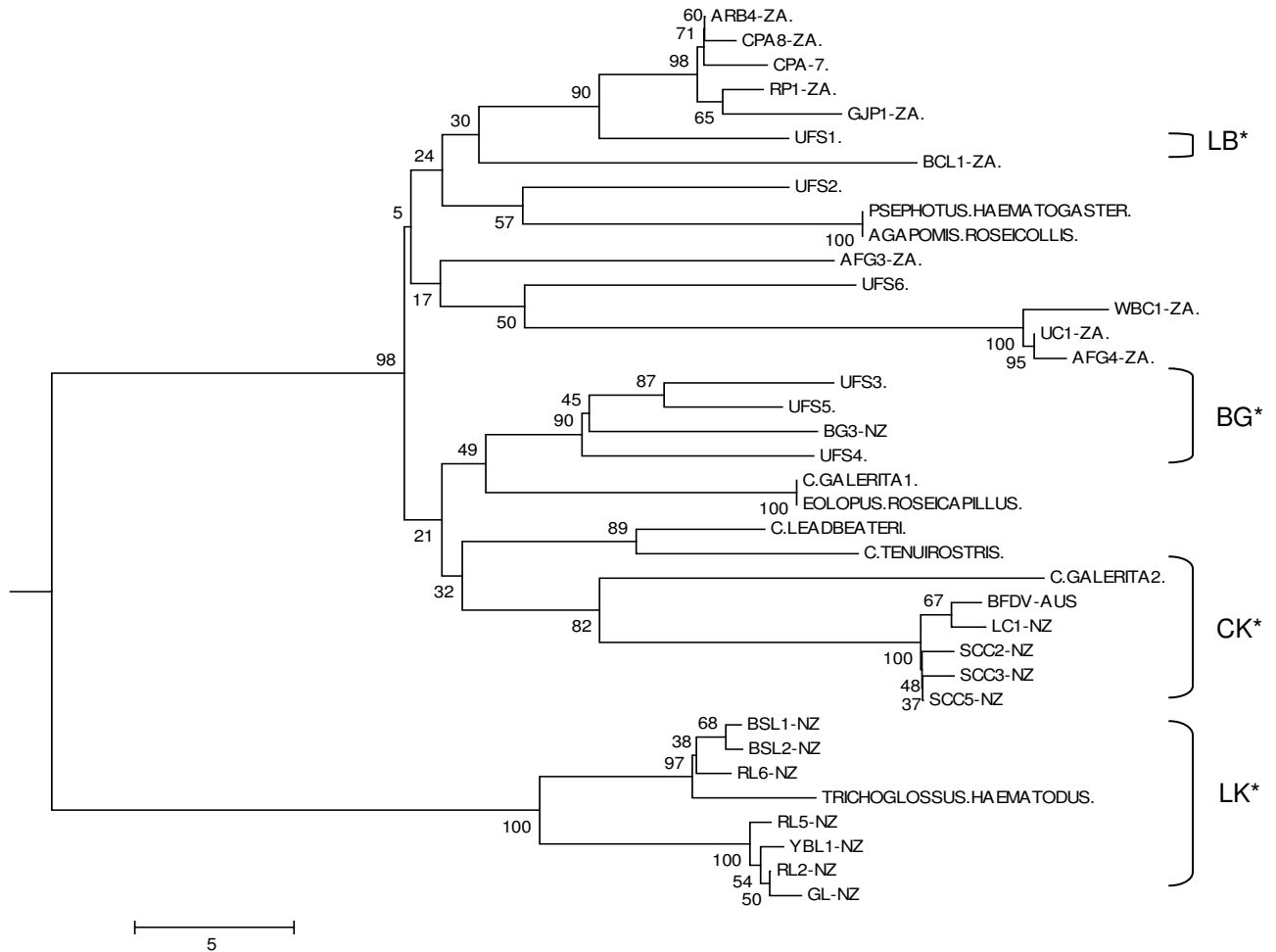
Figure 3.6: A neighbour-joining tree depicting the phylogenetic relationship of the Rep sequences of UFS 1-6 with those of known BFDV isolates. The full length tree was rooted with three non-psittacine avian circoviruses (canary circovirus [GenBank accession number AJ301633], goose circovirus [GenBank accession number AJ304456] and columbid circovirus [GenBank accession number AJ298229]). Only the subtree showing the BFDV isolates and their bootstrap values is shown. The three lineages described by Ritchie *et al.* (2003) are bracketed (CK* [cockatoo], LK* [lorikeet] and BG* [budgerigar]) and refer to the cluster of host species associated with the genotype. The LB* [lovebird] lineage bracketed was reported by Heath *et al.* (2004) and also refers to the species associated with the genotype.

However, the association between genotype and psittacine species is a matter for speculation because this association does not include all the species sampled to date. Isolates such as AFG3-ZA and AFG4-ZA share *Psittacus erithacus* as a

host species but occur in two very different clusters. Furthermore, not all BFDV (SCC1-WA) isolated from cockatoos belong to the CK* lineage.

In addition to these results, the topology of the maximum-likelihood tree based on the Rep sequences differs slightly from that of the tree based on the CP phylogeny (Heath *et al.*, 2004). When the CP is used to reconstruct a tree SCC1-WA (*C.galerita*) is more closely related to BFDV-AUS (*C.galerita*) and forms a cluster that together with Galah-WA (*Eolophus roseicapillus*) and ELBC-SA (*C.tenuirostris*) represents a separate lineage. However, the same isolates in the tree constructed from the Rep sequences cluster differently. SCC1-WA is more closely related to Galah-WA forming a single cluster while ELBC-SA is more closely related to MMC-WA (*C.leadbeateri*) and forms a separate cluster and do not fall in any lineage described by Ritchie *et al.* (2003).

According to Heath *et al.* (2004) the different topologies of the trees are partly due to recombination within Rep. Their study showed evidence of recombination events in all the genomes studied except one. These recombination events occurred in the Rep gene and they suggested that recombination might contribute more to the level of genetic variation within the gene than genetic drift.

UFS 1 formed a cluster on its own and was supported by a high bootstrap value of 90%. In general, a bootstrap value of 70% or above has shown to be a significant support for a cluster association under certain conditions [Hillis & Bull, 1993] and is commonly used as a cut-off value for subtype classification (Wilbe *et al.*, 2003). It was most closely related to the cluster formed by isolates of southern African origin (ARB4-ZA, CPA8-ZA, CPA-7, RP1-ZA and GJP1-ZA). According to Heath *et al.* (2004) this cluster represents a unique genotype in southern Africa and the same might be inferred for UFS 1 as it is supported by a confident bootstrap value.

A similar situation was observed for UFS 6 that was most closely related to isolates WBC1-ZA, UC1-ZA and AFG4-ZA (all southern African isolates). However, a low bootstrap value of 50% did not support its separation into a different cluster. Heath *et al.* (2004) showed these isolates to be closely related to virus isolated in North America and suggested that this genotype may have been recently introduced into southern Africa or that it may represent a fourth genotype that evolved in southern Africa and spread to other parts of the world.

Although UFS 2 appeared to form a separate cluster from its closest relatives (*Psephotus haematogaster* [BB-WA] and *Agapornis roseicollis* [LB-WA]), the bootstrap value of 57% did not support divergence into a new cluster. A 90% bootstrap supported the separation of UFS 4 from the cluster of other budgerigar isolates (UFS 3, 5 and BG3-NZ) and this isolate may represent a separate genotype within the budgerigar lineage. These results do not support any relationship between regional distribution and genetic variation but, the occurrence of a BFDV genotype that preferentially infects budgerigars might be considered.

In a study of C1 gene fragments of BFDV Raue *et al.* (2004), samples from African grey parrot with feather disorders grouped in a separate cluster from those showing immunosuppression. Sequences obtained from an outbreak of acute PBFD in lorikeets also clustered in a separate branch which led them to suggest the possible existence of BFDV genotypes like in the porcine circovirus (PCV). It may be possible that the cluster formed by the budgerigar samples are a representation of a similar incidence and could be a genotype that preferentially infects budgerigars. It may on the other hand represent a genotype that can infect all psittacine species but is only pathogenic in budgerigars. As a result of the small sample size it may be possible that the full extent of BFDV diversity has not been investigated in South Africa thus, UFS 3, 4 and 5 have not been observed in other species and appear to infect budgerigars only. Further

investigation into all these aspects would be important in the study of the molecular biology of BFDV.

While UFS 1-6 are closely related to BFDV isolates in southern Africa, Australia and New Zealand there is a high level of genetic diversity between them. The southern African isolates described by Heath *et al.* (2004) that clustered into three unique genotypes were suggested to represent three separate introductions of BFDV into southern Africa. Considering these three unique genotypes and that UFS 1 may also represent a unique genotype in South Africa, it may be suggested that BFDV was introduced into southern Africa on at least four separate occasions.

The cluster formed by UFS 3, 4, 5 and BG3-NZ showed that these isolates share a most recent common ancestor. This ancestor gave rise to UFS 4 and the cluster of BG3-NZ and UFS 3 and 5 as descendants. The nature of the descendants appears to indicate that this genotype could have possibly evolved in South Africa and then spread to other parts of the world. It represents a similar situation observed by Heath *et al.* (2004) in their isolates (WBC1-ZA, UC1-ZA and AFG4-ZA) and BFDV-USA. A speculation that could be made is that after the divergence of UFS 4, UFS 3 and 5 evolved separately from BG3-NZ, an isolate that could have spread to New Zealand where it was identified.

Ritchie *et al.* (2003) reflected on the role genetic drift plays in the subdivision of BFDV and suggested that the pattern of subdivision in New Zealand could have arisen because of viron transmission along pedigree lines or through single species breeding stocks. Heath *et al.* (2004) identified recombination events in the Rep gene and suggested that it is these events that may contribute more to the genetic variation observed in the Rep gene than genetic drift. In either case, this genetic variation observed in BFDV isolates must continue to be investigated as the genetic diversity of BFDV isolates in different psittacine species may limit the use of PCR as a diagnostic technique.

3.4: CONCLUSIONS.

The method of blood collection used in the study was rapid and less stressful to birds than traditional methods. PCR is a suitable diagnostic test for BFDV but it may be unreliable in diagnosing asymptomatic birds. Six different RFLPs I-VI were observed in a total of 161 samples tested form 19 different psittacine species from six provinces in South Africa.

Sequencing of the ORF 1 amplicon from six representative isolates (UFS 1-6) for the RFLPs I-VI revealed high nucleotide homology to known BFDV isolates. Alignment of both nucleotide and protein sequences of UFS 1-6 showed few differences which re-emphasized the high degree of conservation seen in the Rep gene. Two RCR motifs, the P-loop and pyrophosphatase domain were identified in the protein sequences of UFS 1-6.

A N-J phylogenetic tree depicting the Rep gene sequences of BFDV isolates revealed four lineages, CK*, BG*, LK* and LB*, that have been previously reported. UFS 1 may represent a fourth unique genotype in South Africa and together with the other three genotypes already identified may imply that BFDV was introduced into southern Africa on at least four separate occasions. UFS 3, 4 and 5 isolated form budgerigars cluster with isolate BG3-NZ (identified in New Zealand) and together belong to the BG* (budgerigar) lineage. UFS 6 may belong to the same cluster of southern African isolates that are speculated to have evolved in southern Africa. A high level of genetic diversity exists between each sequence UFS 1-6 as well as between these isolates and known BFDV isolates from southern Africa, Australia and New Zealand which indicates that the diversity of BFDV must be continuously monitored as it may limit the use of PCR in the diagnosis of BFDV infection.

CHAPTER 4.

PURIFICATION OF BEAK AND FEATHER DISEASE VIRUS.

4.1: INTRODUCTION.

Our understanding of the pathogenesis of PBFD (psittacine beak and feather disease) and BFDV (beak and feather disease virus) genome replication has progressed slowly ever since PBFD was first discovered in 1975. This slow growth in the knowledge acquired on a fatal disease like PBFD is due to the inability to propagate BFDV *in vitro* whereby the investigation into the characterization of its proteins (Maramorosch *et al.*, 2001) and the molecular genetics of the virus (Bassami *et al.*, 1998) have been hindered. Attempts have been made to find a suitable cell culture system but to date have been unsuccessful. However, Ritchie *et al.* (1989) managed to purify significant amounts of virus from feather follicle tracts of infected birds which has become one of the most important tools for the study of PBFD.

BFDV has been purified from feather follicle tracts (Ritchie *et al.*, 1989; Latimer *et al.*, 1990), feather dust, faeces, crop washings (Ritchie *et al.*, 1991a), feathers and cloacal contents (Raidal *et al.*, 1993a). Although different methods have been described for the purification, they are all based on zonal centrifugation. The buoyant density of BFDV in caesium chloride (CsCl) is 1.37 g.ml^{-1} which enables the use of CsCl density gradient centrifugation in its purification.

Thus, the objective of this section of the study became an attempt to purify BFDV from different organs of infected birds using CsCl density gradient centrifugation.

4.2: MATERIALS AND METHODS.

All reagents used were chemically pure unless stated otherwise and Appendix A lists the suppliers. Appendix B defines all buffer compositions used.

4.2.1: CsCl DENSITY GRADIENT CENTRIFUGATION.

Body organs such as liver, heart, spleen and kidneys were removed from deceased PBFD positive birds that were housed at the University of the Free State (UFS) and stored at -20 ^{o}C until required. BFDV from individual birds was purified separately according to the methods used by Raidal *et al*. (1993b) with some modifications.

A 10% w/v suspension of organs in phosphate buffered saline [PBS] (pH 7.4) with added 0.5% w/v sodium dodecyl sulphate (SDS) was homogenized on ice using a hand blender (Braun) for 10 minutes (min). The homogenate was filtered and centrifuged (BECKMAN MODEL J2-21 CENTRIFUGE, Rotor JA-20) at 5000 g for 10 min at 4 ^{o}C. Sodium chloride [NaCl] and solid polyethylene glycol [PEG molecular weight (MW) 6 000] (to precipitate the virus) were added to the supernatant to make a final concentration of 1 M NaCl and 10% w/v PEG. The suspension was stirred at 4 ^{o}C for 1 hour and centrifuged at 11000 g for 20 min at 4 ^{o}C to pellet the virus. The supernatant was discarded and the pellet resuspended in a minimal volume of PBS.

Either one of two routes (Figure 4.1) was used to concentrate the virus. Four gradients of CsCl (1.22 g.ml^{-1}, 1.32 g.ml^{-1}, 1.43 g.ml^{-1} and 1.52 g.ml^{-1}) were made in PBS and overlaid in Beckman Quickseal Ultraclear™ Centrifuge Tubes (Beckman). CsCl was added to the sample to make up a final concentration of 1.37 g.ml^{-1} and the sample was layered onto the gradients. Alternatively, two CsCl solutions (1.22 g.ml^{-1} and 1.45 g.ml^{-1}) were made and overlaid and the sample layered onto the gradients without adding CsCl to it. The tubes were

centrifuged (L8 55 Ultracentrifuge, Beckman, Rotor SW 28) at 200 000 g for 24 hours at 4 ^{o}C.

The viral (commonly appearing as an opalescent band) and other fractions were collected by puncturing the side of the tube according to the methods used by Sambrook & Russel (2001). The outer surface of the tube was wiped with ethanol, a piece of Scotch Tape attached to it and an 18-gauge needle was used to puncture the tube through the tape (Figure 4.2). The various fractions were collected into a clean microcentrifuge tube each.

Homogenization of 10% w/v organs
(0.5% w/v SDS, 4 ^{o}C)

Filtration

Centrifugation
(5000 g, 10 min, 4 ^{o}C)

Stirring
(1 M NaCl, 10% w/v PEG, 1 hour, 4 ^{o}C)

Centrifugation
(11000 g, 20 min, 4 ^{o}C)

CsCl density gradient centrifugation
[Sample + CsCl]
(1.22 g.ml^{-1}, 1.32 g.ml^{-1}, 1.43 g.ml^{-1}, 1.52 g.ml^{-1})

CsCl density gradient centrifugation
[Sample – CsCl]
(1.22 g.ml^{-1} and 1.45 g.ml^{-1})

Fraction collection
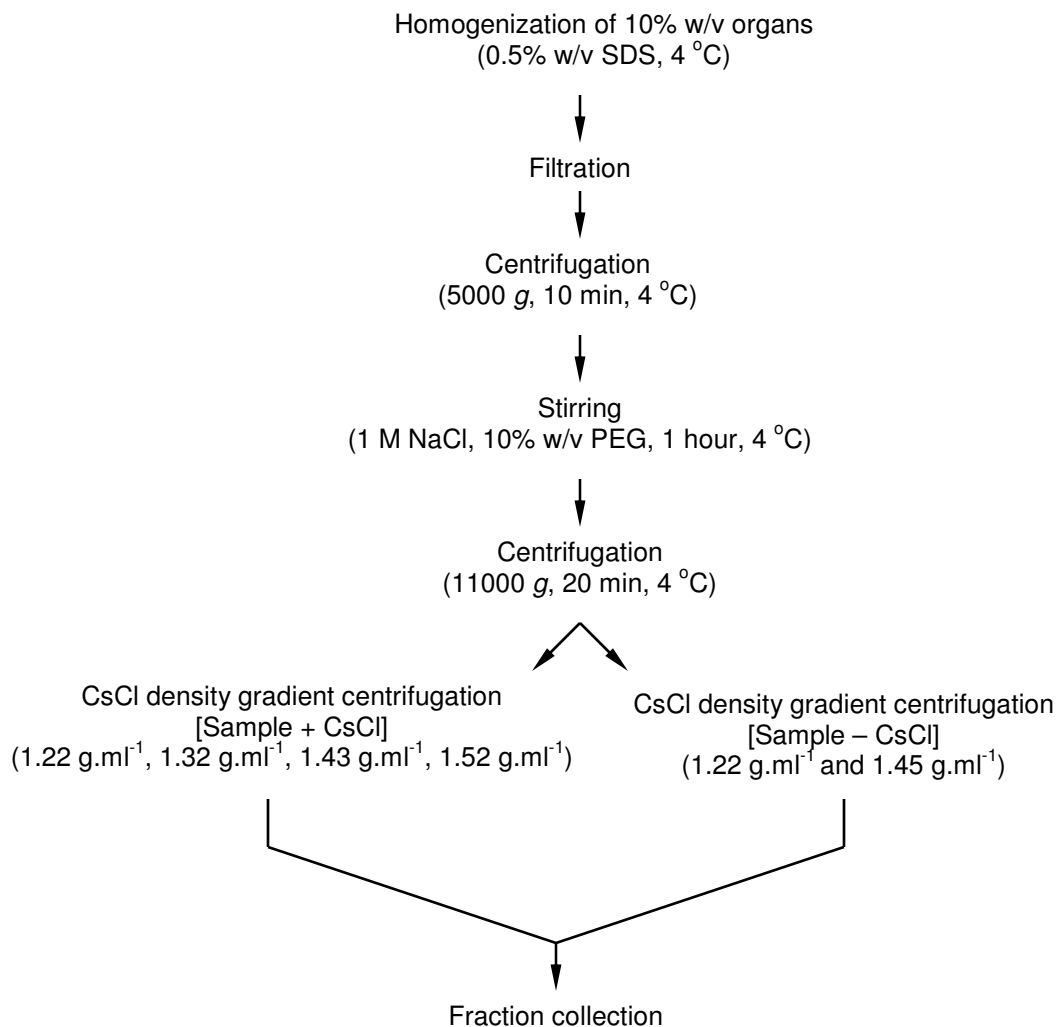
Figure 4.1: A flow diagram indicating the steps followed during the purification of BFDV from body organs of PBFD positive psittacines.

Viral fractions were confirmed by extracting DNA using the QIAamp® DNA Mini Kit (QIAGEN) according to the Blood and Body Fluid Spin Protocol in the manufacturer's instruction booklet and performing PCR as described in Chapter 3.
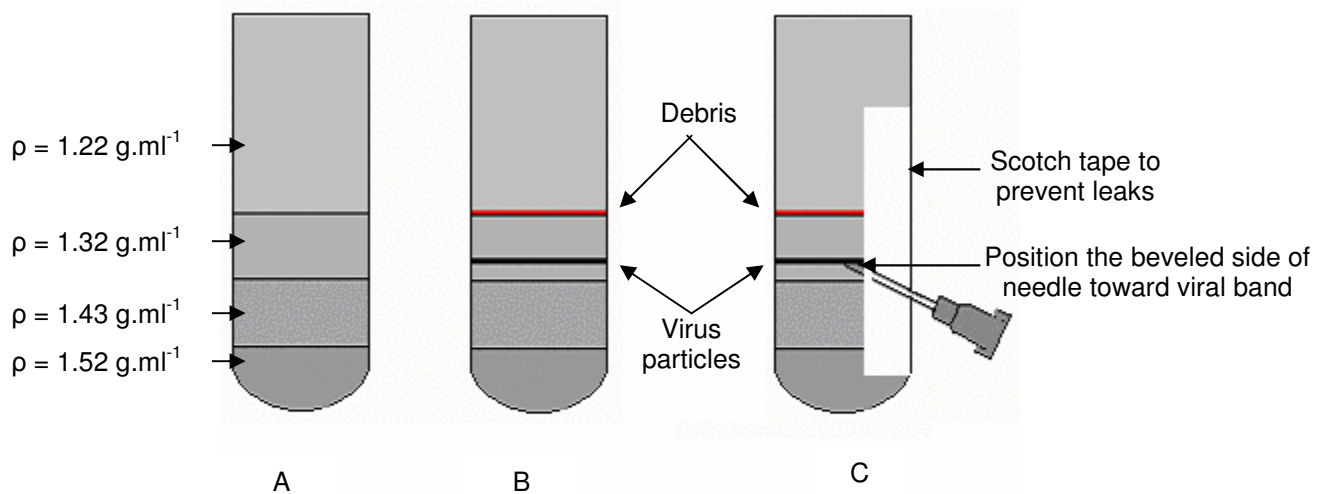
Figure 4.2: CsCl density gradients for purifying BFDV before centrifugation [A] and after centrifugation [B] and the collection of fractions [C] (Sambrook & Russel, 2001).

Proteinase K (20 µl) was pipetted into a clean 1.5 ml microcentrifuge tube. A sample volume of 200 µl was added followed by the addition of 200 µl of Buffer AL. The sample was mixed by vortexing for 15 seconds (s) and incubated at 56 °C for 10 min. The tube was briefly centrifuged, 200 µl of 99.5% ethanol added and the sample mixed by vortexing. After a brief centrifugation step, the sample was applied to a QIAamp spin column in a collection tube and recentrifuged at 6000 g for 1 min. The filtrate was discarded and 500 µl of Buffer AW1 added to the sample. The tube was centrifuged at 6000 g for 1 min, the filtrate discarded and 500 µl of Buffer AW2 added to the sample. After centrifugation at 20 000 g for 3 min, the filtrate was discarded and the column recentrifuged at 20 000 g for 1 min to eliminate buffer carryover. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and 100 µl of Buffer AE applied to it. The

sample was incubated at room temperature (rt) for 5 min, centrifuged at 6000 g for 1 min and the eluted DNA stored at -20 ^{o}C until required. Fractions containing BFDV were also confirmed by haemagglutination (HA) assays (Chapter 5) after a dialysis step.

4.2.2: DIALYSIS OF BFDV CONTAINING FRACTIONS.

A dialysis step was required to remove the CsCl used during centrifugation and was performed using the Slide-A-Lyzer$^®$ Dialysis Cassette (Molecular weight cutoff [MWCO] 10 000, PIERCE). A sample was injected into the cassette and the remaining air removed from the cassette cavity. The cassette was inserted into a buoy and the assembly immersed in one litre of PBS. It was stirred at 4 ^{o}C for at least 4 hours with the buffer being changed twice. After dialysis, the sample was removed from the cavity, filtered through a 0.22 μm membrane filter and stored at -20 ^{o}C until required.

4.3: RESULTS AND DISCUSSION.

CsCl density gradient centrifugation resulted in the purification and concentration of BFDV. Most of the opalescent bands observed that were suspected to contain viral particles tested PCR (Figure 4.3) and HA (Figure 4.4) positive. The use of the Slide-A-Lyzer$^®$ Dialysis Cassettes was a quick and efficient way to remove CsCl molecules from viral fractions of small volumes (0.5-3 ml).

Ritchie *et al.* (1989) used CsCl density gradient centrifugation to purify BFDV and dialysed their fractions before purifying it further. CsCl is considered to inhibit viral infectivity and in a study by Fout *et al.* (2003) it was dialysed out of viral fractions before use in infectivity assays. Ozbun (2002) also dialysed viral fractions to remove CsCl while studying the purification and infection of infectious human papillomavirus type 31b in a cell line. CsCl is also known to inhibit DNA extraction and can be toxic to animals. It is for these reasons that the option of

dialysing out the CsCl was preferred as PCR was used to confirm the presence of virus in the fractions and the purified virus was used to inoculate the rabbits in Chapter 5.
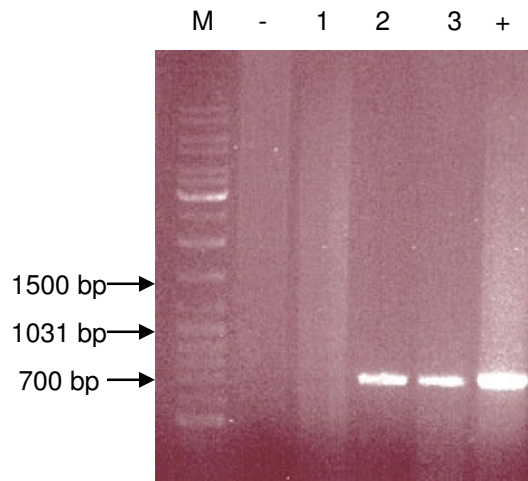
Figure 4.3: A photograph showing negative (Lane 1) and positive (Lanes 2 and 3) fractions amplified by PCR after the purification steps for BFDV from body organs. The PCR amplicon is approximately 700 bp in size. A positive (Lane +) and negative (Lane -) control was also used and the products electrophoresed on a 0.8% w/v agarose gel together with O'GeneRuler™ DNA Ladder (Lane M) as a molecular weight marker.

The PCR test was carried out as discussed in Chapter 3 and was a rapid way of detecting whether the virus was present in the fractions collected. A positive PCR result was supported by a positive HA result but the concentration of viral particles in the fraction could not be distinguished using the PCR technique.

Although CsCl density gradient centrifugation resulted in the concentration of the virus, very low titres were present in the fractions collected. This is indicated in Figure 4.4 where the end-point dilution for HA activity for some fractions was only at a dilution of 1:4 or 1:8 using an initial sample of undiluted virus. This gave a concentration of $\log_2$ 4HA units/100 µl and $\log_2$ 8HA units/100 µl virus particles in the original suspension, respectively. However, this number does not give any

measure of the infectious viral particles present in the suspension but only the amount of haemagglutinating viral particles.
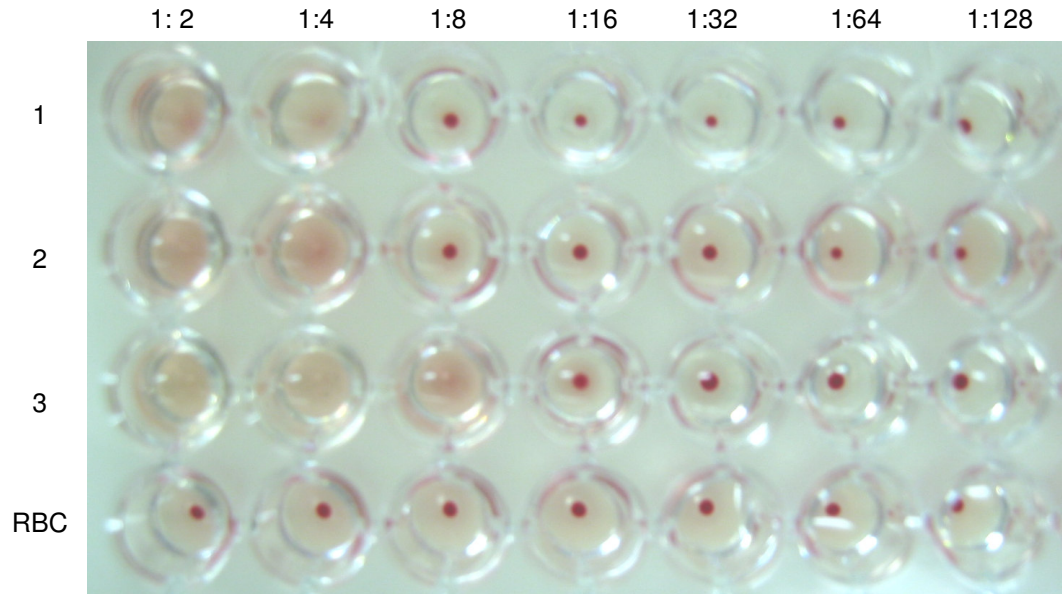
Figure 4.4: Microtitre plate showing haemagglutinating ability of BFDV fractions, purified from infected bird organs by CsCl density gradient centrifugation, with red blood cells (RBCs) from an African grey parrot 959, housed at the UFS. BFDV from each fraction 1-3 was two-fold serially diluted till a 1:128 dilution together with a negative control (Lane RBC) which consisted of RBCs and buffer only. 4 HA units (1:4 dilution) were observed for fractions 1 and 2 while 8 HA units (1:8 dilution) were observed for fraction 3.

The reason for these low viral titres could have been attributed to a possible loss of virus when the supernatant was discarded during the centrifugation steps in the purification. However, when the supernatant was subjected to CsCl density gradient centrifugation, none of the fractions collected yielded either a positive PCR or HA assay result which indicated that the supernatant did not contain any virus which indicated that there was no viral loss being encountered at this stage in the purification.

The absence of virus in the supernatant is as a result of the PEG precipitation method incorporated into the purification protocol. This method has been used by

Fischer *et al*. (1998), Korn *et al*. (2000) and Wu *et al*. (2000) to name but a few research groups in the purification of different viruses. PEG (MW 6000) is added to the supernatant of a viral suspension and stirred for a period of time to allow it to precipitate the virus. A second centrifugation step pellets the virus out of solution and the pellet is finally used in the ultracentrifugation step to purify the virus. Consequently, this probability was eliminated and the purification process was unchanged.

Alternatively, an explanation for these results could stem from the source of the virus used in the purification. Purifications of BFDV from previous studies have mainly focused on the feathers of PBFD positive birds as BFDV is known to be epitheliotropic in feathers and feather follicles. Ritchie *et al*. (1991a) recovered BFDV from feather dust, faeces and crop washings and found a higher concentration of virions in the feather dust than in the faeces. Crop washings also yielded low concentrations of virus. In their study it was observed that virus was recovered in faeces and crops of birds that had substantial feather dystrophy but their statistical analysis found no significant difference in virus recovery that could be attributed to the degree of feather and beak lesions.

Raidal *et al*. (1993b) found high HA titres in the liver of chronically affected birds that suggested hepatic disease. Riddoch *et al*. (1996) observed high concentrations of BFDV in the faeces of PBFD-affected birds and suggested that this could be due to the excretion of the virus from the liver in the bile. BFDV has been identified in numerous tissues including the liver, kidneys and spleen but it may be possible that the titres in the organs used in these experiments were minimal, which could explain the low concentrations of virus purified. A study by Johne *et al*. (2004) tested a crude virus preparation extracted from the liver and spleen of a budgerigar in an HA assay but did not present the results for the assay so a comparison of their titres to those in the present study can not be performed.

Ritchie & Carter (1995) reported that in some recovered birds as the feathers were replaced with new and healthy feathers BFDV could not be detected in the blood or feathers. However it is unknown if some birds that show signs of recovering might not have completely eliminated the virus from their systems and instead are asymptomatic carriers. Another consideration is that PBFD-affected neonatals that survive for years in a featherless state may have diminished levels of BFDV circulating in them. The birds used for viral purification in the present study had survived for a number of years in a featherless state before succumbing to the disease and it can be suggested that these birds may have had low levels of BFDV present in them. BFDV is known to be epitheliotropic in the feathers and follicles, targeting replicating cells within the basal layer of the epithelium (Latimer *et al*., 1991) and once the bird becomes bald or featherless it may present an unfavourable habitat for the virus. Although the mechanism of infection is unclear, the disease is a dermatologic condition and the feathers play an important role in the life cycle of this virus. This would imply that a loss of feathers could be interpreted as an interruption or may be even a complete stop in the life cycle of the virus which would result in the decreased levels of virus present in the bird as shedding continues.

Although a measure of the concentration of purified virus is relevant, it was difficult to quantify the viral particles as the HA assay which is quantitative for BFDV was not available in South Africa. This subsequently led to part of the current study being to establish a working HA assay (Chapter 5) and thus, volumes instead of viral concentration have been cited in the report.

The volumes of purified viral suspensions were also minimal usually accumulating to less than 1 ml per purification run which limited the number of experiments carried out as well as the amount of research that could be performed. These volumes would have been sufficient had it not been necessary to use undiluted virus in the HA and HI assays (Chapter 5). Additionally, the availability of infected organs for purification purposes was also limited as the

study relied on donations from South African bird owners. Some of the birds that had tested positive once in the testing period between January 2003 and May 2004 (Chapter 3) indicated negativity for BFDV when a retest was performed and could not be used for purification. Positive birds that are still alive have been kept for other research purposes and so purifications could only be carried out using the few deceased birds that remained positive upon their death.

Purification of BFDV using CsCl density gradient centrifugation is quite a tedious process that is time consuming thus, emphasizing the need to find a cell culture system for the cultivation of this virus which would enable more in depth research to be carried out.

4.4: CONCLUSIONS.

BFDV could be successfully purified from organs like the liver, spleen and kidneys using CsCl density gradient centrifugation although only in small volumes (purified viral concentrations could not be estimated due to lack of a quantitative HA assay). The viral titres obtained were low which could be attributed to the source of virus or the state of the birds used in the purification. CsCl molecules were removed from viral fractions using Slide-A-Lyzer® Dialysis Cassettes which are rapid and efficient enabling the use of PCR and HA assay to confirm the presence of BFDV. The PCR test and HA assay established in the present study were reliable tests that could be used to confirm BFDV in purified samples.

CHAPTER 5.

ANTIBODY PRODUCTION AND ESTABLISHMENT OF A SEROLOGICAL DIAGNOSTIC TEST FOR BEAK AND FEATHER DISEASE VIRUS.

5.1: INTRODUCTION.

Purified viral preparations have been used to raise antibodies to beak and feather disease virus (BFDV) in rabbits (Latimer *et al.*, 1990; Ritchie *et al.*, 1990) and in chickens (Raidal *et al.*, 1993a) and have already been used in serological diagnostic tests like immunohistochemical staining and haemagglutination inhibition (HI) assays. Monoclonal antibodies have been raised in mice (Ritchie *et al.*, 1992b) that were tested using an enzyme-linked immunosorbent assay (ELISA) among other serological tests, although a commercially available ELISA has not yet been established. These antibodies have the distinct benefit of allowing recovery of virus from infected tissues by affinity purification techniques which will aid the further characterization of BFDV proteins. They could also be used to develop a standardized test for the accurate diagnosis of virus-induced lesions and aid in the detection of virus-infected cell cultures where the cytopathic effects may be minimal.

An important factor to consider when raising antibodies in laboratory animals is the use of immunologic adjuvants. These are agents that non-specifically increase the immune responses to specific antigens that are weakly immunogenic (Jennings, 1995) but are also known to induce some harmful side effects in inoculated animals. Some of these side effects include discomfort, anorexia, weight loss, lethargy and underlying irritation at the site of inoculation.

Two common adjuvants used are Freund's complete and incomplete adjuvants [FCA and FIA, respectively]. FCA is a water-in-oil emulsion of mineral oil, mannide monooleate and heat-killed *Mycobacterium tuberculosis* or components of the organism that stimulates both humoral and cell-mediated immunity (Jennings, 1995). It preferentially induces antibody against epitopes on denatured proteins but because of its toxicity, its use in laboratory animals is limited. This toxicity arises due to the inability of the animals to metabolise the mineral oil as well as the ability of the mycobacterial elements to elicit severe granulomatous reactions.

On the other hand FIA does not contain *M.tuberculosis* or any of its cell components and is thus less toxic. However, it is less effective in inducing high antibody titres and enhancing cell-mediated immunity than FCA. Thus, FCA is normally administered only for initial immunizations or when weakly immunogenic antigens are used followed by booster immunizations in FIA.

Diagnosis of psittacine beak and feather disease (PBFD) relies on the detection of viral nucleic acid by polymerase chain reaction (PCR) or the detection of viral antigen and antibody by the haemagglutination (HA) and HI assay respectively. The HI assay is a rapid, quantitative serologic test that is used for detecting exposure to BFDV by detecting BFDV-specific antibodies. A bird exposed to BFDV can be fatally infected or the bird will mount an immune response. Low antibody titres detected in a bird could be suggestive of the presence of clinical disease but a high titre could be an indication of a bird's immunologic response that can clear away the infection.

Although the erythrocyte suitability for use in the test varies between species as well as among individuals of a species, HI assays have been applied successfully in detecting routes of BFDV shedding from infected birds (Ritchie *et al.*, 1991a) and in monitoring antibody titres during vaccination challenge studies (Ritchie *et al.*, 1992a). These assays could also be a mechanism for determining

seroprevalence of BFDV antibodies in both wild and captive psittacines as well as an important tool in epidemiological studies.

Sanada & Sanada (2000) reported on the ability of BFDV to haemagglutinate erythrocytes of the guinea pig, Goffin's cockatoo, Galah, eastern long-billed Corella, sulphur-crested cockatoo, Major Mitchell's cockatoo and goose. In South Africa the HA and HI assays are unavailable as a diagnostic test because most of the psittacine species especially the Galah are not easily available as they are not indigenous to Africa. In addition to the birds being expensive to acquire not all individuals of a species are suitable sources of erythrocytes for the BFDV HA assay (Sanada & Sanada, 2000).

It thus became the aim of this section of the study to attempt to successfully raise antibodies to BFDV in rabbits and use these antibodies together with purified virus (Chapter 4) to establish an HA and HI assay in South Africa for the detection of PBFD. A second objective was to find suitable, locally available erythrocytes to use in these assays as a substitute for Galah erythrocytes (commonly used in Australia).

5.2: MATERIALS AND METHODS.

All reagents used were chemically pure unless otherwise stated and their suppliers are listed in Appendix A. Appendix B defines all buffer compositions used.

5.2.1: INOCULATION OF RABBITS.

BFDV that was purified from an Eclectus parrot using caesium chloride (CsCl) density centrifugation and confirmed to contain virus by PCR was used to inoculate two rabbits, A and B. The same viral fraction was used in all booster inoculations. An 18-gauge needle was used in the inoculations and the syringe

was filled with 200 µl of purified BFDV (viral concentration could not be estimated due to lack of a quantitative HA assay) that was mixed with 200 µl of FIA as the adjuvant. Air bubbles were removed from the syringe and the thigh area of the rabbit disinfected. The rabbits were inoculated intramuscularly (IM), each time in the thigh area. Two booster inoculations comprising the same volume of virus and adjuvant as the primary inoculant were administered 2 and 4 weeks later, respectively.

Prior to the primary inoculation, blood was obtained from the rabbits as a negative control. The ear of the rabbit was disinfected and rubbed with xylene to dilate the veins. It was pricked with a butterfly needle and approximately 1 ml of blood was collected into a microcentrifuge tube without any anticoagulant. Blood was obtained from each rabbit one week after the last booster inoculation as afore mentioned to test for the presence of antibodies. The blood samples that were collected were allowed to clot overnight in order to retrieve serum. The serum was stored at -20 ^{o}C until required.

5.2.2: COLLECTION AND CONCENTRATION OF ERYTHROCYTES.

Three African grey parrots with ring band numbers 956, 957 and 959 (housed at the University of the Free State [UFS]) that had tested PBFD negative (Chapter 3, Table 3.4) over a period of two years and five Brown-headed parrots were used to check for erythrocyte suitability in an HA assay (see section 5.2.4 for more details). Blood from each species was collected into Alsever's solution (refer to Appendix B for buffer compositions) to prevent coagulation and pooled into two fractions: African grey parrot and Brown-headed parrot erythrocytes. Additional HA assays were performed using erythrocytes obtained from either one of the three African grey parrots.

Concentration of the erythrocytes was carried out immediately after collection according to Villegas (1989) with slight modification. Blood was centrifuged at

440 g for 4 min and the supernatant discarded. The erythrocytes were washed 3 times by resuspending the sample to the original volume using phosphate buffered saline (PBS) free from calcium [Ca^{2+}] and magnesium [Mg^{2+}] ions and centrifuging at 440 g for 4 min each time. After the last washing, the erythrocytes were resuspended in the wash buffer (PBS – Ca^{2+}, Mg^{2+}) to make a final concentration of 25% w/v stock solution. The solution was stored at 4 ^{o}C until required and only for a maximum period of seven days. A 0.75% w/v working solution was made up immediately before use in the HA and HI assays.

5.2.3: PREPARATION OF GLUTARALDEHYDE FIXED RED BLOOD CELLS (RBCs).

In an attempt to prolong the working span of the erythrocytes, African grey parrot number 959 RBCs were fixed using glutaraldehyde [GA] according to Eaves *et al.* (1989). A total of 0.5 ml of African grey parrot blood was collected into Alsever's solution (50:50 final ratio) and the cells centrifuged at 775 g for 15 min. They were washed three times with 0.15 M NaCl (515 g for 5 min) and the packed cell volume estimated. A 1-2% w/v solution of the RBCs was incubated in a glutaraldehyde-salts solution (refer to Appendix B for composition) for 30 min at 4 ^{o}C then centrifuged at 515 g for 10 min. The cells were washed five times with 0.15 M NaCl and five times with distilled water. The sample was suspended in distilled water to make up a 30% w/v suspension that was stored in small amounts at 4 ^{o}C in the dark until required. A 1% w/v solution was made up immediately before use in the HA assay where the application of the GA-fixed RBCs was tested.

5.2.4: HAEMAGGLUTINATION ASSAY (HA).

The HA assay was performed by slightly modifying that used by Ritchie *et al.* (1991b). The test was performed in 96-well plastic V-bottom microtitre plates (greiner) and was first used to test whether erythrocytes from African grey parrots

or Brown-headed parrots could be haemagglutinated by BFDV. Subsequent tests were performed to confirm purified fractions of BFDV (Chapter 4).

A volume of 50 µl of PBS buffer containing Mg^{2+} and Ca^{2+} ions (HI buffer) was added to each well. Thereafter, 50 µl of undiluted BFDV was added to the first well, mixed carefully by pipetting up and down and two-fold serially diluted. This was done by taking 50 µl of suspension from the first well and placing into the second well. The suspension in the second well was mixed in the same way as previously described and 50 µl removed from it and placed in the third well. This process was continued until the last well after which 50 µl was removed form this well and discarded leaving a total volume of 50 µl in each test well. For the negative control, no BFDV was added to the wells while the positive control lane consisted of a known positive BFDV fraction. 50 µl of 0.75% w/v erythrocytes was added to each well and mixed by pipetting slowly up and down. The plate was incubated at room temperature (rt) for 30 to 45 min to allow the erythrocytes to settle completely and then read.

5.2.5: HAEMAGGLUTINATION INHIBITION ASSAY (HI).

Each time an HI assay was performed, an HA assay was simultaneously carried out in order to validate the specificity of the reaction. Due to low viral titres present in the fractions (Chapter 3), undiluted virus was used in the HI assay.

A volume of 50 µl of HI buffer was added to each well followed by the addition of 50 µl of serum which was subsequently two-fold (or ten-fold in some instances) serially diluted in the same manner as described for the HA assay. Serum tested was obtained from three African grey parrots (numbers 967, 968 and 970) housed at the UFS that had previously tested positive and then negative by PCR (Table 3.4) as they were suspected of having antibodies against BFDV. Serum from rabbits A and B prior to inoculation and post inoculation, serum from rabbits that were not inoculated, two fractions of pooled chicken serum and heat-

inactivated rabbit serum were also tested. Rabbit serum was heat inactivated by incubating 200 µl of serum at 56 °C for 30 min. The serum was then diluted 1:40 in PBS and absorbed with 0.2 volumes of 25% w/v RBC overnight at 4 °C.

A volume of 50 µl of undiluted virus was added to each well containing buffer and serum and the plate incubated at rt for 30 min. Thereafter, 50 µl of 0.75% w/v erythrocytes was added to each well, the plate incubated at rt for a further 45 min and read. The negative control wells did not have BFDV added to them. Serum from the three African grey parrots numbered 967, 968 and 970 that had tested positive for antibodies were subsequently used as a positive control.

5.2.6: ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

All buffers were made up immediately before use for the ELISA. Costar high-binding ELISA plates were coated with 100 µl of purified BFDV that was diluted 1:4, 1:8 and 1:16 in carbonate buffer (50 mM, pH 9.6). Four wells were used as negative controls and were left uncoated. The plates were covered with parafilm to retain moisture and incubated overnight at rt. Thereafter the plates were washed three times with 200 µl washing buffer [1X TST] (50 mM Tris, 150 mM NaCl, 0.05% v/v Tween 20, pH 8.0). All the wells were blocked for one hour at 37 °C with 300 µl per well of blocking buffer (5% w/v ELITE non-fat milk powder dissolved in 1X TST) and washed again with 1X TST.

Rabbit A and B serum before and after inoculation was diluted 1:2, 1:10, 1:20 and 1:100 in the blocking buffer. Each serum was added to the wells in 100 µl volumes and for the wells coated with a 1:8 and 1:16 dilution of virus the assay was performed in duplicate. Two-fold serial dilutions of the sera could not be used in the assay due to the limited amounts of purified virus available. A volume of 100 µl of a 1:2 dilution of rabbit A and B serum before and after inoculation was also added to the four control wells. The plate was incubated at 37 °C with shaking for 1 hour and the washing step repeated. Goat anti-rabbit peroxidase

conjugate (Sigma) was diluted 1:5000 in blocking buffer and 100 µl was added to all the wells. The plate was again incubated at 37 ^{o}C for 1 hour with shaking. Substrate in the form of 4,4,5,5'-tetramethylbenzidine [TMB] (1 TMB tablet dissolved in citric acid, Na_2HPO_4 and H_2O_2) was added to all the wells in 100 µl volumes and the enzyme reaction allowed to develop for 10 minutes. The reaction was stopped by adding 50 µl of 2N H_2SO_4 and the absorbance read at a wavelength of 450 nm.

5.3: RESULTS AND DISCUSSION.

5.3.1: ERYHTROCYTE SUITABILITY AND THE USE OF GA-FIXED RED BLOOD CELLS (RBCs) IN HA ASSAYS.
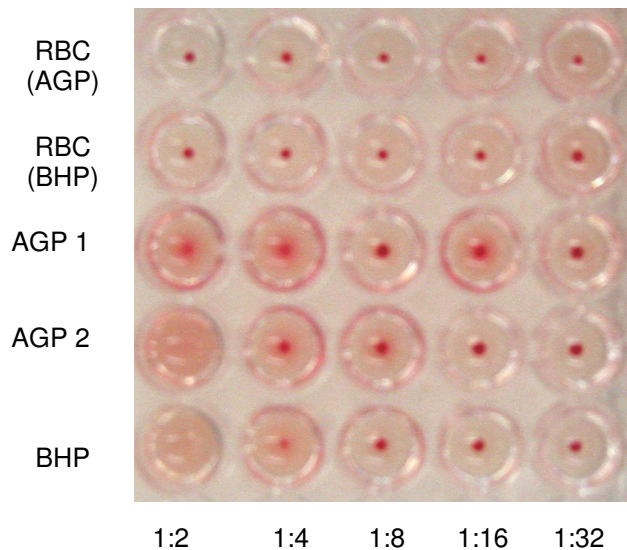
Figure 5.1: Microtitre plate indicating haemagglutinating activity of BFDV with RBCs from a pooled fraction of African grey parrot blood [Lane AGP 2] and its negative control [Lane RBC (AGP)] and with erythrocytes from a pooled fraction of Brown-headed parrot blood [Lane BHP] and its negative control [Lane RBC (BHP)]. Two-fold serial dilutions were performed till a 1:32 dilution while the negative controls consisted of RBCs and buffer only. Lane AGP 1 represents the partial haemagglutination reaction observed when RBCs from the pooled African grey parrot blood were incubated with a fraction that contained very low titres of BFDV.

The two pooled fractions of African grey parrot and Brown-headed parrot erythrocytes demonstrated haemagglutinating activity with a known fraction of purified BFDV particles (Figure 5.1). In addition, when erythrocytes from either one of the three African grey parrots were used individually, haemagglutinating activity was also observed (Figure 5.2). These results indicate the ability of BFDV to haemagglutinate erythrocytes from both African grey parrots and Brown-headed parrots, both indigenous species to Africa and thus more easily available and economical for use in both HA and HI assays in South Africa.

Erythrocytes from African grey parrots and Brown-headed parrots have not been previously reported to have haemagglutinating activity with BFDV. Unlike previous reports concerning variability in erythrocyte suitability from the same psittacine species (Sanada & Sanada, 2000), no substantial differences in haemagglutinating activity were found between individual African grey parrots when erythrocytes were used separately (Figure 5.2).

The search for sources of erythrocytes that have haemagglutinating ability with BFDV for use in the HA assays have been numerous. Many countries do not have indigenous birds that can be used as a source of erythrocytes and most of these birds that have haemagglutinating activity are Australian in origin which makes them quite expensive in other countries especially in Africa. Soares *et al*. (1998) tested the use of Amazon parrots and macaws as a source of erythrocytes but their results showed that erythrocytes from these Brazilian birds did not have haemagglutinating activity in the presence of BFDV.

Such birds are New World psittacines and it has been suggested that these species are less likely to be infected by BFDV than Old World psittacines. Ritchie & Carter (1995) reported that DNA probe testing indicates that Old World Psittaciformes are most likely to be infected as they are more susceptible and more likely to develop feather abnormalities than New World Psittaciformes. They further went on to give some statistics of birds tested by DNA probing

whereby 30% of lovebirds, 10.2% of Eclectus parrots, 8.7% of cockatoos and 8% of African grey parrots were positive while only 4% of macaws and Amazon parrots indicated virus presence.
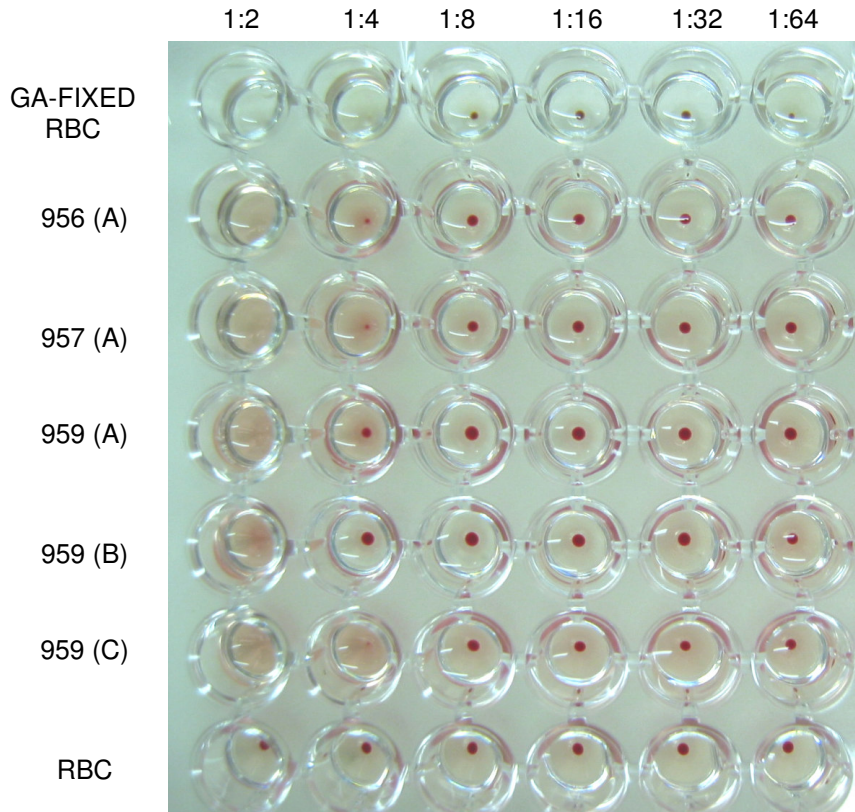
Figure 5.2: Microtitre plate indicating the use of GA-fixed RBCs in the HA assay. 2 HA units each were observed when viral fraction A was incubated with RBCs from African grey parrot blood obtained from birds 956, 957 and 959 [Lanes 956 (A), 957 (A) and 959 (A), respectively]. No substantial differences in haemagglutinating activity were observed between RBCs obtained from the individual parrots. Lanes 959 (B) [2 HA units] and 959 (C) [4 HA units] represent haemagglutinating activity of RBCs from African grey parrot 959 with different fractions B and C of purified BFDV. The negative control [Lane RBC] consisted of RBCs and buffer only.

It would appear that there may exist a relationship between haemagglutinating activity with BFDV and susceptibility to disease. The ability of BFDV to haemagglutinate erythrocytes from African grey parrots and Brown-headed

parrots shown in this study further serves to peak interest in the existence of such a relationship and further investigation into this area would be warranted.

The use of GA-fixed RBCs in the HA assay was successful as haemagglutination was observed up to a 1:4 dilution when tested with a known fraction of BFDV (Figure 5.2). No differences in haemagglutinating activity were observed which indicates that the use of GA-fixed RBCs would be more suitable to use than non-fixed RBCs. Johne *et al.* (2004) also used GA-fixed RBCs in their HA and HI assays and even went further by blocking their plates to prevent non-specific binding with no report of any drawbacks in their research. The reason for suggesting the use of GA-fixed RBCs instead of non-fixed RBCs is that it would be less stressful to the birds as GA-fixed RBCs have a longer working life. Only a small volume of blood can be drawn at a time from both the species used in this study and time has to be allowed for the regeneration of blood if non-fixed RBCs are used. This limits the amount of research as well as prolonging the time for diagnosis of birds (if carried out commercially) that can be done within a certain time period. Use of GA-fixed RBCs would be a more economical option that would be animal sensitive as well.

5.3.2: ESTABLISHMENT OF THE HA ASSAY.

The HA assay was successfully established using erythrocytes from African grey parrots and Brown-headed parrots and was used to test numerous fractions for the presence of BFDV (Figure 5.2). Samples that tested positive in the PCR diagnostic test for BFDV infection exhibited corresponding positive results for haemagglutinating activity in the HA assays. Due to these correlating results, the HA assay was used in this study to confirm PCR results for the purified BFDV fractions.

Most of the fractions yielded between 2 (1:2) and 8 (1:8) HA units for the end-point dilution which indicated that the purified fractions had very low titres of

BFDV. As a result of these low titres, any subsequent reaction could best be performed using undiluted viral fractions.

Ritchie *et al.* (1991a) used the HA assay to determine the routes and prevalence of shedding of BFDV and reported on the high HA titres of virus found in the feather dust and faeces of PBFD affected birds, confirming transmission through these routes. The HA assay established in this study could be used for the same purpose to test the birds housed at the UFS. Swab samples of nucleic acid from these areas could be taken and tested by PCR and the results compared to determine if PCR can also be used to perform some epidemiological studies where the HA assay is not available.

The HA assay established in the study was economical and rapid as locally available erythrocytes could be used in the test. The only drawback was that the source of erythrocytes could not handle the load of assays required to be done but this problem was solved by the use of the GA-fixed RBCs as discussed in section 5.3.1. It also served as a measure for the purified BFDV fractions and a good confirmatory test for BFDV antigen when diagnosing PBFD by PCR.

However, the HA assay itself can also be used as a suitable serological diagnostic test for BFDV infection in South Africa. Riddoch *et al.* (1996) reported on the use of the HA assay to detect BFDV in feathers, tissues and faeces which is sensitive and specific. The use of such samples would allow the easy and rapid diagnosis of BFDV infection in South African psittacines and the cost of performing an HA would be less expensive than a PCR diagnostic test. Although the HA assay can not detect latent or incubating BFDV infection and from the present study PCR on blood samples from asymptomatic birds may not be as reliable as was originally suggested (Chapter 3), a combination of these two diagnostic tests might increase the chances of detecting BFDV infection in both symptomatic and asymptomatic birds.

5.3.3: ESTABLISHMENT OF THE HI ASSAY.

With the establishment of a functioning HA assay, the validity of the HI assay was tested successfully by performing a simultaneous HA assay each time an HI assay was carried out. However, due to the limited volumes of purified virus which was available as well as the low concentration of purified BFDV in the study, serial two-fold dilutions to test for antibodies to BFDV could not be performed and the end-point dilution could only be estimated as a range and not an exact measure of HI titre.

The initial HI assay carried out was performed with a viral fraction containing 2 HA units and serum from three African grey parrots (numbers 967, 968 and 970) suspected to carry antibodies to BFDV. A 1:2 dilution showed the presence of haemagglutinating inhibition activity (Figure 5.3a) thus, three dilutions (1:10, 1:20 and 1:100) were used to estimate the end-point titration of the original undiluted serum sample which lay between the 1:20 and 1:100 dilution (Figure 5.3b). The exact value of the end-point would have been a good indication of the concentration of antibodies present in the birds as the HI is also the only quantitative method available for detection of BFDV specific antibodies (Sanada & Sanada, 2000). This inhibition of haemagglutination indicated the presence of antibodies in the serum from the three African grey parrots which may account for the negative test results observed during the testing schedule after they had tested positive (Chapter 3, Table 3.4). At the time of testing the bird may have just been exposed to the virus and was picked up by PCR as it is a very sensitive test but was able to mount an immune response and clear away the infection by the second test date. The serum from any of the three birds was then used in subsequent HI assays as a positive control.

The level and type of antibodies required for a bird to be protected from BFDV infection is presently unknown but the antibody titre observed in the African grey parrots in this study are sufficient to indicate that these birds that show no clinical

symptoms of PBFD are constantly being exposed to the virus shed in feather dust and faeces by infected birds housed with them. Ritchie *et al.* (1991b) reported that the demonstration of HI titres in clinically normal birds that were being naturally exposed to BFDV substantiated the suspicion that birds in contact with PBFD affected birds are possibly being continually exposed to BFDV shed in feather dust and faeces.

(A)   (B)
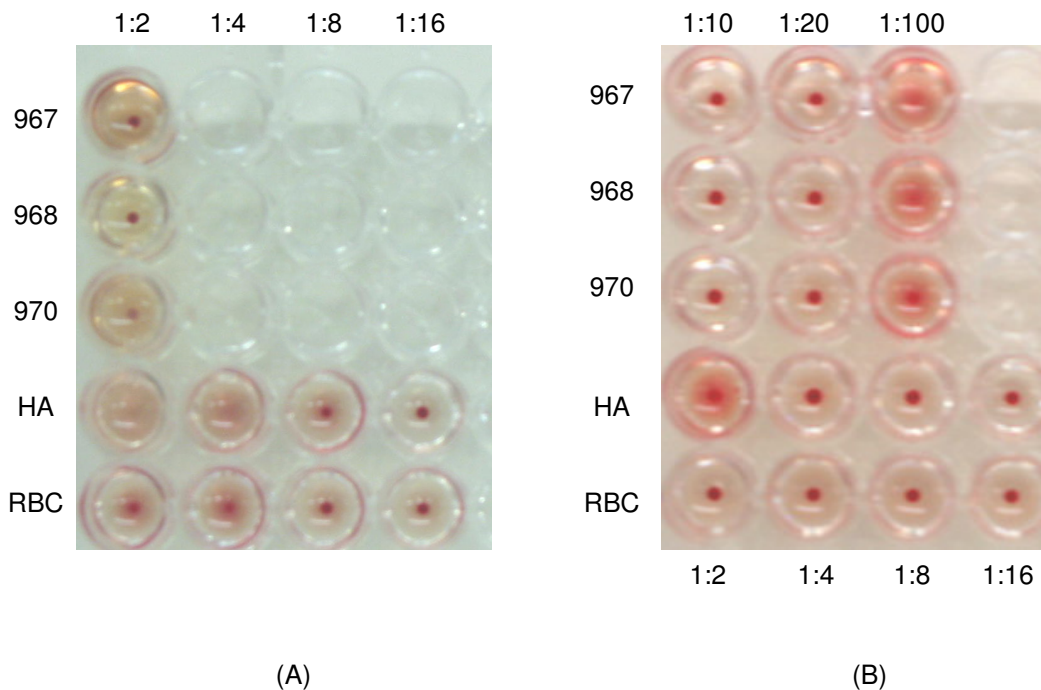
Figure 5.3: Microtitre plates showing haemagglutination inhibition by BFDV antibodies present in African grey parrot serum 967, 968 and 970 [Lanes 967, 968 and 970, respectively] at dilutions of 1:2 (A), 1:10, 1:20 and 1:100 (B).The end-point titration lies between the 1:20 and 1:100 dilution (B). An HA assay [Lane HA] was performed simultaneously up to a 1:8 dilution (A) and 1:16 dilution (B) for each HI assay. The negative control consisted of RBCs and buffer only [Lane RBC].

Ritchie *et al*. (1991b) also reported that the presence of HI titres would suggest that the birds in contact with PBFD affected birds were being exposed to sufficient quantities of virus to mount an immune response. However, it is unknown whether the titres in this study are sufficient enough to prevent BFDV

infection as repeated tests of some of the birds (Chapter 3, Table 3.4) indicate variable PCR results. A good follow-up test would be to obtain serum from the birds that had variable PCR results and determine whether the HI titres present in these birds are low. Low titres demonstrated in infected birds may be explained by the severe damage that occurs to the bursa and thymus and/or by the apparently persistent infections that occur in macrophages (Ritchie *et al.*, 1991b).

With adequate quantities of virus, the HI assay established in this study could be used to detect the presence of antibodies to BFDV in serum samples and quantify them. The HA assay for detection of BFDV in feathers , tissues and faeces is sensitive and specific provided that the observed haemagglutination is inhibited by BFDV-specific antibody (Riddoch *et al.*, 1996). Together with the HA assay established in the study, the HI assay could also be used as a rapid and economical serological diagnostic test for BFDV infection in South Africa by confirming the HA results of a diagnosis. It can also be used to detect BFDV specific antibodies in bird that would indicate exposure to the virus. The only shortcoming of using the HI assay is that there are serious problems in the standardization and interpretation of the results (Johne *et al.*, 2004).

5.3.4: HI RESULTS FOR RABBIT SERUM PRE AND POST INOCULATION.

The inoculation of the rabbits with FIA did not produce any discomfort to the animals or adverse reactions like irritation at the injection site. Adverse reactions are a major consideration with any vaccine containing mineral oil and those that cause undesirable tissue reactions are unacceptable (Raidal *et al.*, 1993b). FCA is normally used as the adjuvant in the primary inoculation especially as the general rule is that it should be used only for weakly immunogenic antigens and only for initial immunizations (Jennings, 1995). The use of FIA as the adjuvant in both the primary and secondary inoculations was decided upon as FCA is very toxic and reports have shown that not only severe systemic reactions and local

ulcerations may occur but also that the immune response to antigens can be decreased rather than increased (Jennings, 1995). The detection of antibodies in the inoculated rabbits (refer to section 5.3.5 for details) indicates that BFDV is a strong immunogenic antigen as it stimulated antibody production even though it was administered in FIA as an adjuvant.

Currently only one strain of BFDV has been identified and comparison of recovered viruses from a sulphur-crested cockatoo, a Black Palm cockatoo, a Red-lored Amazon parrot and a Peach-faced lovebird revealed morphologically and antigenically similar isolates (Ritchie & Carter, 1995). However, due to the inability to propagate BFDV *in vitro* minimal investigation has been performed on the antigenicity of the virus and for this reason the rabbits were inoculated with purified virus from a single Eclectus parrot. This ensured that the antibodies raised did not differ antigenically.

Rabbit serum before and after inoculation, when tested in the HI assay together with African grey parrot serum as a positive control, yielded positive HI results for all the serum samples prior to inoculation and for serum sample B after inoculation and haemagglutination for serum sample A post inoculation. These results were unexpected as they indicated that rabbits A and B had been previously exposed to BFDV and had already developed antibodies prior to inoculation. To exclude this factor, a second HI assay was performed using serum obtained from newly acquired rabbits that had been guaranteed free from exposure to BFDV. The same results were obtained and haemagglutination inhibition was recorded for the negative serum samples (Figure 5.4). Additionally, PCR tests on the rabbit sera pre inoculation yielded negative results for BFDV thus confirming that rabbits A and B were not harbouring the virus.
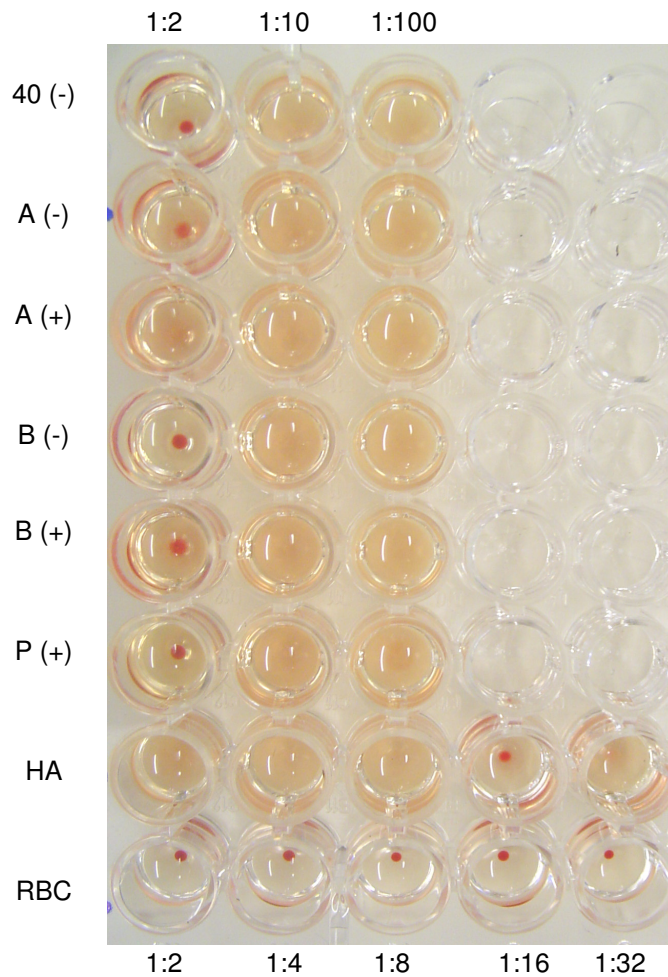
Figure 5.4: Microtitre plate showing the HI assay results for different sera. Serum obtained from a rabbit that had not been previously exposed to BFDV [Lane 40 (-)], rabbits A and B pre inoculation [Lanes A (-) and B (-), respectively] and rabbit B post inoculation [Lane B (+)] all showed haemagglutination inhibition activity at a 1:2 dilution and haemagglutination at 1:10 and 1:100 dilutions of sera. Serum from rabbit A post inoculation indicated haemagglutination at 1:2, 1:10 and 1:100 dilutions of serum [Lane A (+)]. The positive control used was parrot serum [Lane P (+)] that only showed haemagglutination inhibition at a 1:2 dilution of serum. Lane HA represents the HA assay performed simultaneously with the HI assay and lane RBC is the control lane that consisted of buffer and RBCs only.

The presence of a non-specific factor in the rabbit serum may be responsible for the false-positive inhibition observed as similar results were repeatedly observed. This factor may be able to bind to both antigen and antibody, preferentially binding to antibody, which would explain why in negative serum samples

inhibition is observed (factor bound to antigen) and in positive samples haemagglutination is observed (factor preferentially bound to antibody). This is not a novel event as Raidal *et al*. (1993a) and Sexton *et al*. (1994) have already reported on the occurrence of non-specific reactions in chicken and cockatoo serum respectively, due to HA and/or HA inhibitors that cause false-positive results. Non-specific serum HA might be viral antigens, serum components or antibodies with affinity for Galah erythrocytes (Raidal *et al*., 1993a) and this may be the case for African grey erythrocytes as well.
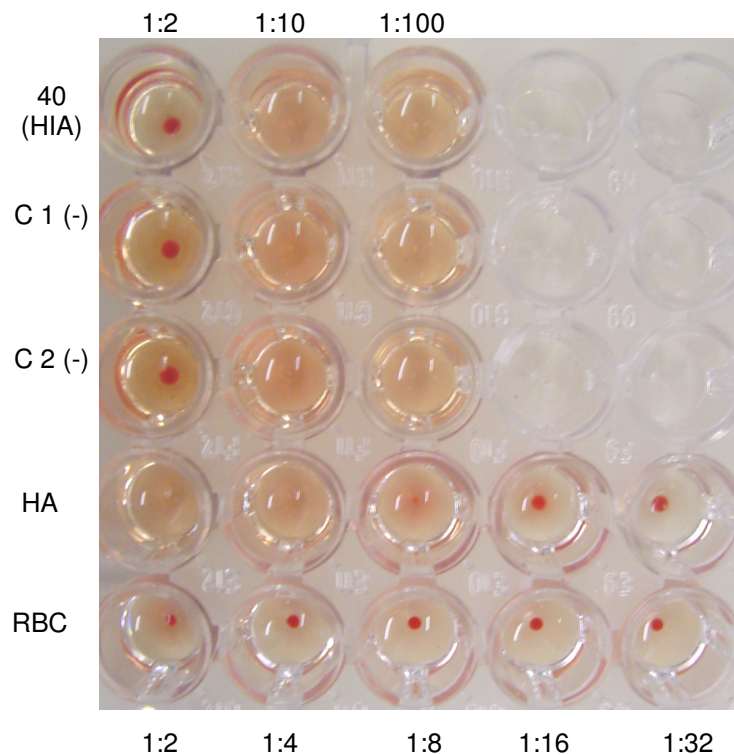
Figure 5.5: Microtitre plate indicating the haemagglutination inhibition observed for heat inactivated rabbit serum [Lane 40 (HIA)], and two fractions of chicken sera [Lanes C 1 (-) and C 2 (-), respectively] at a dilution of 1:2 and haemagglutination at a 1:10 and 1:100 dilution of the sera. Lane HA represents the HA assay carried out simultaneously with the HI assay and lane RBC represents the negative control lane that consisted of buffer and RBCs only.

To further investigate whether there was a non-specific factor present, 1:10 and 1:100 dilutions of the serum samples were used in the HI in an attempt to dilute out the factor. Theoretically it was expected that the antibody titres present in the rabbit serum post inoculation were high enough to still bind to antigen and result in inhibition at higher dilutions as compared to a lesser concentration of the non-specific factor which would then be diluted out. However, the expected results were not obtained and HI was observed in all the samples including the negative sera at a 1:2 dilution (Figure 5.4).

To verify the presence of the proposed non-specific factor in rabbit serum, two fractions of chicken serum (with no prior exposure to BFDV) were diluted to 1:2, 1:10 and 1:100 and tested in the HI assay to distinguish between sera from the two species but the same results were observed for the chicken serum (Figure 5.5). This indicated that in addition to rabbit, chicken serum may also contain such a factor that causes non-specific reactions in HI assays and that this occurrence might be observed in all non-psittacine sera.

Rabbit serum that was heat inactivated in an attempt to eliminate such non-specific factors was also inhibited thereby indicating that this factor could not be removed by heating. However, Raidal *et al*. (1993a) and Sexton *et al*. (1994) also reported on the inability to heat inactivate the HA and/or HA inhibitors that they came across. Further investigation into the characterization of such a factor is necessary to determine what type of molecule it is, how it interacts with antigen and antibody and how to eliminate such non-specificity for the HI assay to become a reliable serologic test when using non-psittacine sera.
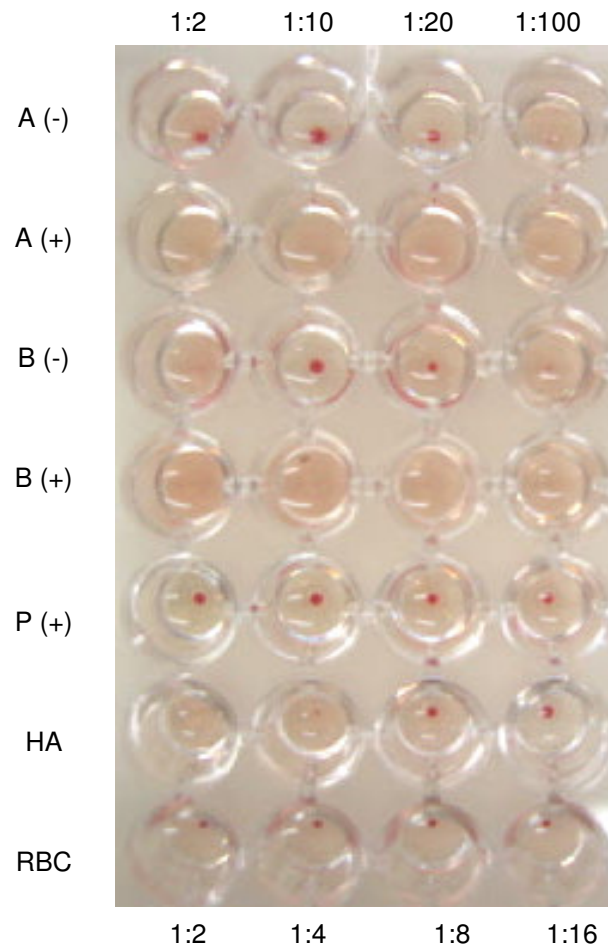
Figure 5.6: Microtitre plate of an HI assay indicating haemagglutination inhibition for rabbit serum samples A and B pre inoculation [Lanes A (-) and B (-), respectively] and haemagglutination for rabbit serum samples A and B post inoculation [Lanes A (+) and B (+), respectively]. The positive control lane [P (+)] consisted of parrot serum and exhibited inhibition up to a 1:100 dilution of serum. An HA assay [Lane HA] was performed simultaneously with the HI assay and the negative control [Lane RBC] was made up of buffer and RBCs only.

Although a non-specific factor could be present in the parrot serum as well, since Sexton *et al.* (1994) reported the presence of non-specific haemagglutinins in cockatoo sera, the low HI observed in the positive control was contrary to the results obtained in the first HI assay where parrot serum exhibited an end-point dilution between 1:20 and 1:100 (Figure 5.3b). It was then realized that different fractions of purified virus had been used in this HI assay (8 HA units) as compared to the first HI assay (2 HA units) carried out and this could have led to

the results observed. An HI assay performed with the same serum samples at a dilution of 1:2, 1:10, 1:20 and 1:100 using a BFDV fraction of 2 HA units yielded inhibition for rabbit serum A and B pre inoculation, haemagglutination for serum A and B post inoculation and inhibition for the positive control up to a 1:100 dilution (Figure 5.6). These results still indicated the occurrence of a non-specific reaction in the rabbit serum samples but none in the parrot serum.

With these conflicting results and the occurrence of non-specific reactions, a more efficient serological test than the HI assay was required to detect BFDV-specific antibodies in sera which led to the attempt to establish a working ELISA.

5.3.5: ESTABLISHMENT OF A DIRECT ELISA.

The enzyme reaction of the ELISA developed an intense yellow colour in the wells that contained serum from rabbits A and B post inoculation at 1:4, 1:8 and 1:16 viral dilutions (except for B that was not tested at a 1:16 dilution) although the yellow colour became less intense at a 1:16 dilution of virus than at 1:4 and 1:8 dilutions [Figure 5.7 (I,II and III, respectively) , lanes A (+) and B (+)]. The wells that contained serum obtained prior to inoculation did not develop such an intense colour, appearing almost transparent [Figure 5.7 (I, II and III, respectively), lanes A (-) and B (-)]. These results confirm that BFDV-specific antibodies were successfully raised in the rabbits after inoculation with BFDV purified in this study.

The control wells also appeared transparent [Figure 5.7 (IV)] indicating that the wells were sufficiently blocked. This prevented non-specific binding of antibody to the wells and resulting in a false-positive reaction. If the wells are not blocked it is possible that the rabbit raised antibodies would bind to the walls of the well and then in turn be bound by the anti-rabbit conjugate antibodies. This would result in the development of a colour reaction that would be interpreted as a false-positive reaction. Furthermore, the differences in absorbance (A_{450}) readings (Table 5.1)

between the wells containing rabbit serum pre inoculation and post inoculation also indicated that the colour observed was not due to binding of non-specific antibodies.

1:2   1:10   1:20 1:100 1:2   1:10   1:20 1:100   1:2   1:10   1:2   1:10
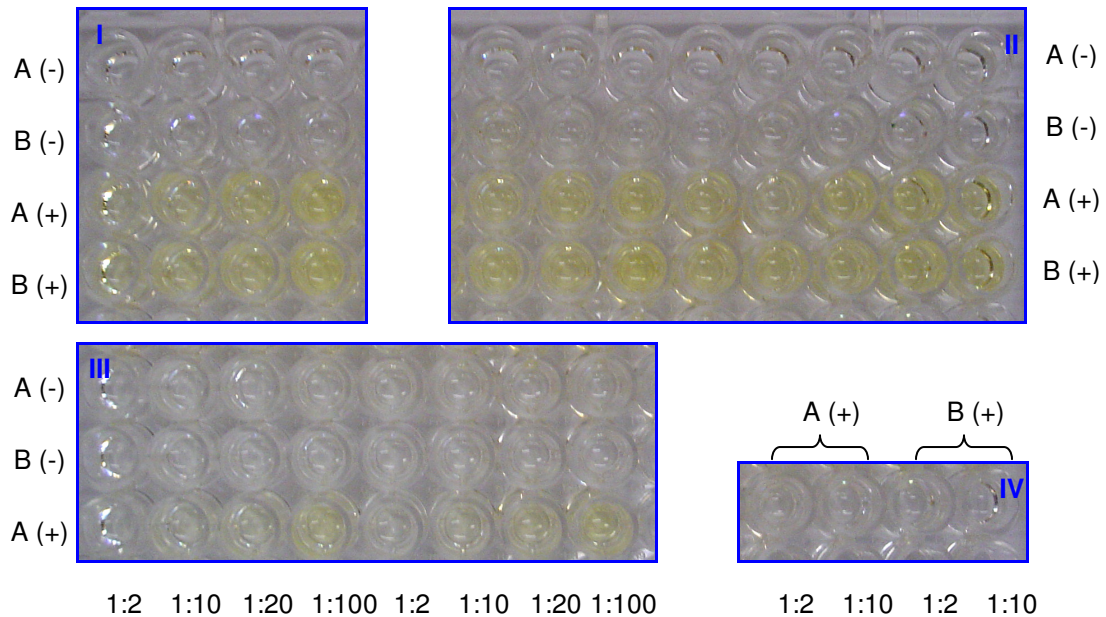
Figure 5.7: Sections from an ELISA plate showing the absence and presence of antibodies in rabbit serum pre [Lanes A (-) and B (-)] and post [Lanes A (+) and B (+)] inoculation, respectively, at 1:4, 1:8 and 1:16 dilutions of purified BFDV (I, II and III, respectively). The yellow colour indicates the presence of BFDV specific antibodies [Lanes A (+) and B (+)] at dilutions of 1:2, 1:10, 1:20 and 1:100 of serum. Section IV represents a set of negative controls with uncoated wells containing serum diluted at 1:2 and 1:10 from rabbits A and B post inoculation. The lack of an intense yellow colour in these wells indicates that non-specific binding of antibodies which could result in false-positive reactions did not occur yielding the assay valid.

Readings for BFDV antibody positive sera did not differ much between the 1:2, 1:10, 1:20 and 1:100 dilutions as these dilutions were low. If a quantitative measure of the antibodies was required, a higher dilution, for example 1:500, would be more suitable.

Table 5.1: Absorbance readings for different dilutions of rabbit A and B serum pre and post inoculation in an ELISA with purified BFDV.

			Serum dilutions	BFDV dilutions			
				1:4	1:8	1:16	Uncoated
Average absorbance readings (A_{450})	Pre inoculation	A	1:2	0.082	0.085	0.085	
			1:10	0.069	0.078	0.087	
			1:20	0.072	0.081	0.091	
			1:100	0.070	0.091	0.107	
		B	1:2	0.102	0.140	0.083	
			1:10	0.145	0.098	0.082	
			1:20	0.079	0.157	0.079	
			1:100	0.092	0.106	0.082	
	Post inoculation	A	1:2	0.214	0.197	0.113	0.079
			1:10	0.220	0.278	0.150	0.079
			1:20	0.275	0.305	0.183	
			1:100	0.317	0.256	0.247	
		B	1:2	0.194	0.293		0.084
			1:10	0.228	0.293		0.082
			1:20	0.252	0.344		
			1:100	0.319	0.299		

As a limited amount of purified BFDV was available, the assay could not be carried out in duplicate for all the viral dilutions (1:4) nor could two-fold serial dilutions of serum be performed. Consequently a titration curve could not be constructed to obtain an optimum antigen range for application in the ELISA. However, the aim of establishing an ELISA in this study was only for the benefit of verifying the presence of BFDV-specific antibodies raised in the rabbits as this could not be confirmed using the HI assay.

Johne *et al.* (2004) developed an ELISA and immunoblotting assay using a recombinant truncated capsid protein as antigen for the detection of BFDV antibodies in psittacine sera that correlated well with their HI results. Further studies should include the standardization and optimization of this direct ELISA to establish it as a commercial serologic diagnostic test for BFDV infection both

nationally and internationally. An important consideration would then be to resolve the standard concentration of virus used to coat the wells and the dilution factor used for the serum to be tested which can be obtained from the titration curves. However, it has to be taken into account that no 'gold standard' exists for the serological detection of BFDV infection (Johne *et al.*, 2004).

5.4: CONCLUSION.

African grey parrots and Brown-headed parrots provide a local and economical source of RBCs for application in the HA and HI assays to detect BFDV antigen and antibody, respectively, in South Africa. GA-fixation of RBCs, which prolongs their working life span, can be used to preserve the erythrocytes from African grey parrots and does not result in any functional differences compared to non GA-fixed cells when applied in the HA assay. The HA assay was successfully established and is a rapid, quantitative serological test that can be used to confirm the presence of BFDV in samples that have tested positive by PCR. The HI assay set up in this study was used to confirm the presence of BFDV-specific antibodies in African grey parrot sera. Together with the HA assay, it can be used as a reliable serological diagnostic test for BFDV infection. However, due to a factor which cannot be eliminated by heat inactivation, but causes non-specific reactions in the HI assay, it is not a suitable tool for use in the detection of BFDV-specific antibodies in non-psittacine sera.

A crude direct ELISA for the detection of antibodies confirmed the production of BFDV-specific antibodies in rabbits A and B that had been inoculated with a fraction of BFDV in FIA which was purified in this study. As a result of its reliability in the detection of antibody in non-psittacine sera, the ELISA set up in this study must be standardized to establish it as an efficient diagnostic test for BFDV infection.

CHAPTER 6.

GENERAL DISCUSSION AND CONCLUSIONS.

Psittacine beak and feather disease (PBFD) is a major problem in psittacine birds worldwide that is now threatening the extinction of some of these species like the Cape parrot which is already an endangered species. This dangerous situation has augmented the need to develop a vaccine for the protection of parrot chicks against the disease. It therefore became the objective of this study to establish techniques that could be used to work towards such a vaccine. An important aspect to consider is to determine if there is one or more strains of beak and feather disease virus (BFDV), like porcine circovirus (PCV), so that protection to all strains can be accomplished. The possible occurrence of such strains can be ascertained by investigating both genetic and antigenic differences in the known BFDV isolates which is carried out using molecular and serological techniques that have been attempted in the current study.

Investigation into the genetic diversity of BFDV isolates in South Africa revealed five different restriction fragment length polymorphisms (RFLPs) previously reported (RFLP I-V) (Albertyn *et al.*, 2004) and one (RFLP VI) identified in the present study that indicated the presence of genetic variation in the region of the Rep gene studied. Of the six RFLPs, RFLP I was common to all 19 psittacine species tested. RFLP II was obtained from a single sample and perhaps if more samples had been submitted for testing from the same area it would have been observed more often. RFLP III, IV and V were all observed in budgerigars only although other samples obtained from budgerigars also presented the profile of RFLP I. RFLP VI was found in a number of species that also showed RFLP I but these samples were obtained from a single breeder and this pattern appeared to be confined to this particular farm. All these differences encouraged the use of phylogenetic analysis in an attempt to clarify the genetic variation observed in this region of open reading frame 1 (ORF1).

The sequences UFS 1-6 were representative of the RFLPs I-VI and refer to the region within ORF 1 that was amplified by PCR and sequenced. Both a nucleotide and protein alignment of these sequences revealed high homology between the sequences. Two of the rolling circle replication (RCR) motifs, the P-loop sequence and the pyrophosphatase domain were identified from the protein sequence alignment. The high homology of the protein sequences and the presence of the different motifs for RCR corroborate the high level of conservation previously reported in the Rep gene.

However, a neighbour-joining (NJ) tree rooted with three non-psittacine avian circoviruses indicated the occurrence of genetic diversity between UFS 1-6 as well as between known BFDV isolates. The cockatoo [CK*], lorikeet [LK*], budgerigar [BG*] (Ritchie *et al.*, 2003) and lovebird [LB*] (Heath *et al.*, 2004) lineages were identified in the tree as well as the three unique genotypes identified by Heath *et al.* (2004).

UFS 3, 4 and 5 clustered with the isolate BG3-NZ that was identified in a budgerigar in New Zealand. This isolate was used to define the BG* lineage by Ritchie *et al.* (2003) who suggested an association between genotype and psittacine species. Isolates UFS 3, 4 and 5 also identified in budgerigars fall within this lineage as they cluster with BG3-NZ thereby reiterating the situation observed by Ritchie *et al.* (2003). However, the relationship between genotype and psittacine species is speculative as many isolates with the same host species do not fall into the designated lineages, especially isolates from the cockatoo species. In addition to this, isolates from host species other than cockatoo, lorikeet, budgerigar and lovebird do not cluster in their respective species to form lineages of their own and are quite diverse from each other for example the isolates AFG3-ZA and AFG4-ZA both from the host *Psittacus erithacus*.

A second interesting result was the cluster formed by UFS 1(representative of RFLP I that appeared common in South Africa). The high bootstrap value (90%) separated this isolate from other southern African isolates that it was most closely related to and indicates it to be a unique genotype. If this is true for UFS 1 it would imply that there are four unique genotypes in southern Africa including the three identified by Heath *et al.* (2004). These four genotypes would then represent the introduction of BFDV into southern Africa on at least four separate occasions.

The cluster formed by UFS 3, 4 and 5 and BG3-NZ may represent a similar situation observed by Raue *et al.* (2004) who reported on the possible existence of BFDV genotypes. Their study indicated that two clusters that were formed, one by African grey parrots suffering from feather disorders and the other by lorikeets suffering from acute PBFD, may infer the existence of such genotypes. If the budgerigar isolates are characteristic of such a circumstance it may be possible that there exists a genotype of BFDV that preferentially infects budgerigars or may even be pathogenic only in this species. A large enough number of samples (161 in the study) was not tested to give an indication of whether such a genotype exists but it is interesting to note that RFLP III, IV and V (represented by UFS 3, 4 and 5) were only found in budgerigars from the 19 different species tested.

Although UFS 1-6 are closely related to known BFDV isolates from southern Africa (UFS 1 and 6), Australia (UFS 2) and New Zealand (UFS 3, 4 and 5), the different clusters formed indicate the presence of a high level of diversity between these isolates. The reason for this genetic diversity has been attributed to genetic drift (Ritchie *et al.*, 2003) and recombination events within the Rep gene (Heath *et al.*, 2004).

The purification of BFDV from body organs using cesium chloride (CsCl) density gradient centrifugation was a successful attempt. Polymerase chain reaction

(PCR) and haemagglutination (HA) assays were both used to confirm the presence of viral particles in the fractions collected and the two results correlated very well. A fraction that yielded a positive PCR result also showed the occurrence of HA activity of red blood cells (RBCs) by BFDV. Although PCR is a good indication of the presence of viral particles it is not a quantitative test and this is supplemented by using the HA assay which is currently the only method for detecting BFDV that is also quantitative (Sanada & Sanada, 2000).

HA assays indicated that only a very low concentration of virus was obtained from the various virus purification runs. Initially, it was supposed that virus was being lost in the supernatant that was discarded after the second centrifugation step after the addition of polyethylene glycol (PEG). A purification run employing the supernatant in the ultracentrifugation step yielded no fraction with BFDV particles but a purification run using the resuspended pellet resulted in the purification of the virus. The reason for this is that the PEG precipitation method employed in the process allowed the virus to be precipitated and the second centrifugation step led to the pelleting of the precipitated virus.

It is suggested that these low concentrations of virus obtained ranging from 4 to 8 HA units could be as a result of the body organs used or the bird from which the organs were obtained. Low levels of virus may be present in the body organs of infected birds as the virus is known to be epitheliotropic in the feathers and follicles (Latimer *et al.*, 1991) but a more likely reason is that at the time of its death, the bird which had survived with PBFD for a number of years, may have had low concentrations of BFDV circulating in it. It was established during this work that the birds housed at the University of the Free State (UFS) had developed detectable antibodies against BFDV and this could also contribute to the low concentrations of virus purified in these experiments.

In addition to the low concentrations of virus, each purification run yielded small volumes of purified virus. Due to the low concentrations of virus, techniques like

the HA and haemagglutination inhibition (HI) assays and the enzyme-linked immunosorbent assay (ELISA) which would commonly employ virus in a diluted form, had to be performed with undiluted virus fractions. The limited supply of organs also played a role in the low supply of virus for use in the serological techniques.

The HA and HI assays in the study were established using RBCs obtained from African grey parrots and Brown-headed parrots. BFDV has the ability to haemagglutinate RBCs from a number of psittacine species [galahs (Latimer *et al.*, 1994) and cockatoos (Johne *et al.*, 2004) being most commonly used as their RBCs are more sensitive for detection of BFDV than other avian species]. However, it is necessary to choose suitable erythrocytes because the HA activity of BFDV differs for erythrocytes of different species and the variability in erythrocyte suitability from the same species has been reported by Sanada & Sanada (2000).

The HA assay using RBCs from pooled African grey parrot blood or individual parrot blood did not differ in the results obtained. Additionally, the use of glutaraldehyde (GA)-fixed RBCs was also successful. GA-fixed RBCs have a more prolonged working span and provide a more suitable option to bleeding birds on a regular basis. This can be quite stressful and traumatic for the birds as well as limiting because of the need to let the birds regenerate their lost blood cells.

A study by Soares *et al.* (1998) on the use of RBCs from Brazilian birds (amazons and macaws) in the HA assay proved unsuccessful. These species belong to the New World psittacines and are reported to be less susceptible to BFDV infection than Old World Psittaciformes (Ritchie & Carter, 1995). This susceptibility to infection may be related to the use of RBCs from different psittacine species in the HA assay with the Old World psittacines being the more suitable source of RBCs. Both African grey parrots and Brown-headed parrots

belong to the Old World Psittaciformes and it may be for this reason that they were found to be suitable as sources for RBCs.

The HI assay was used to test a number of sera for the presence of BFDV specific antibodies. Three African grey parrots were suspected to have antibodies to BFDV because they were in constant contact with PBFD-affected birds and their PCR test results indicated that they were negative. HI assay results confirmed this suspicion as HI was observed up to a 1:20 dilution of the sera. This indicates that the birds, showing no clinical symptoms, had produced antibodies against BFDV and supported the suggestion by Ritchie *et al.* (1991b) that the presence of HI titres in birds in contact with PBFD affected birds indicated that they were being exposed to sufficient quantities of virus to mount an immune response. This is also a good indication that the eventual production of a vaccine will be successful.

Serum from rabbits A and B inoculated intramuscularly in the thigh with equal volumes of purified virus in equal volumes of Freund's incomplete adjuvant (FIA), were tested in the HA assay. Serum from the rabbits before inoculation indicated the presence of HI while serum collected after inoculation, that was supposed to contain antibodies to BFDV, showed the occurrence of HA activity. These results were contradictory as serum before inoculation was supposed to indicate HA because the absence of antibodies would allow BFDV to agglutinate the RBCs and form a lattice on the top, while serum after inoculation was supposed to have BFDV specific antibodies that would bind to the virus preventing it from agglutinating the RBCs. This would result in the RBCs falling to the bottom of the well to form a button; indication that HI had occurred.

It was suspected that the rabbits may have been previously exposed to BFDV thus, sera from newly acquired rabbits was obtained and also tested in the HI assay. Similar results were observed; negative sera showed HI activity. This led to the speculation of the presence of a non-specific factor in the rabbit serum that

could react with the virus. To determine if such a factor was only present in rabbit serum, sera obtained from chickens (never been exposed to BFDV) was tested in the HI assay. The same results were obtained, and the ability of the virus to agglutinate the RBCs was inhibited by negative chicken serum. Attempts to inactivate the factor by heating at 56 ^{o}C for 30 minutes proved fruitless as once again similar results were achieved.

However, such a situation is not inimitable as Raidal *et al.* (1993a) and Sexton *et al.* (1994) both reported on the occurrence of non-specific reactions in chicken and cockatoo serum, respectively, due to HA and/or HA inhibitors that cause false-positive results. To ensure that the inoculated rabbits had not been previously exposed to BFDV, the serum obtained before inoculation was tested by PCR and the results were negative for BFDV nucleic acid; an indication that the rabbits had not been harbouring the virus prior to inoculation.

In an attempt to verify the presence of rabbit raised antibodies, which could not be done with the HI assay, due to apparent non-specific inhibitors, an ELISA test was set up. An intense colour reaction developed in serum obtained post inoculation in the wells coated with a 1:4, 1:8 and 1:16 dilution of BFDV. The negative sera had very little or no colour reaction which indicated the absence of antibodies. Rabbit raised antibodies in the serum bind to the virus coated on the wells. The secondary antibody (in this study, goat anti-rabbit IgG peroxidase) used to detect the primary antibody, in turn binds to it and when substrate (4,4,5,5'-tetramethylbenzidine) for the peroxidase is added, product formation is identified by the colour reaction that occurs and its absorbance can be read at a wavelength of 450 nm.

In order to ascertain that the colour reaction that occurred was due to the presence of BFDV-specific antibodies, four control wells were used. These were left uncoated, without virus, but were blocked to prevent non-specific binding of antibody. Little or no colour reaction developed confirming that the colour

reaction in the serum obtained after inoculation was due to rabbit raised BFDV specific antibodies. A false-positive reaction would have been indicated if a colour reaction had developed in the control wells. This is because if non-specific rabbit antibodies had bound to the walls of the well, they would have still been detected by the secondary antibody conjugate and a colour reaction would have developed.

Future work would include investigation into the genetic diversity of BFDV. A number of lineages and especially genotypes have been identified but the entire extent of the genetic diversity in BFDV has not been fully investigated. The occurrence of recombination in the Rep gene indicates a high level of diversity present in BFDV and this further supports the need for in depth investigations. The need to propagate the virus *in vitro* intensifies as more investigation into the genetic diversity would be helped along if the virus could be cultured.

Studies seem to indicate that there is only one strain of BFDV worldwide but it may also be possible that like PCV and TTV, more strains of the virus exist but have not been detected as yet. The clustering of BFDV isolates into separate groups indicates genetic variation in this virus but it is unclear whether this variation denotes differences in the antigenicity and pathogenicity of each known isolate. The antibodies raised in this study can be used in antigenic studies of different isolates.

The ELISA established in the study can be refined and used in the antigenic studies. It would present the ideal serological test, using the rabbit-raised antibodies to detect purified virus samples obtained from a number of psittacine species. Investigation into potential genetic and/or antigenic differences would be necessary for the development of a vaccine that would be able to protect chicks against any isolate(s) of BFDV.

In conclusion, UFS 1-6 are closely related to other BFDV isolates but a high level of genetic diversity exists between them. UFS 1 may represent a unique genotype in South Africa and together with three other unique genotypes identified in southern Africa by Heath *et al.* (2004) indicates that BFDV was introduced into southern Africa on at least four separate occasions. UFS 3, 4 and 5 belong to the budgerigar lineage identified by Ritchie *et al.* (2003) as they cluster with isolate BG3-NZ. Purification of BFDV by CsCl density gradient centrifugation can be done using body organs as a viral source. However, the titres obtained in the study were low and could be attributed to low levels of virus present in the organs of PBFD-affected birds or to the low levels of circulating virus in a bird that is dependent on the state of that bird. African grey parrots and Brown-headed parrots are a local, economical and easily available source of RBCs for use in the HA and HI assays in South Africa. The HA and HI assays established in the study can be used as serological diagnostic tests for BFDV and can also be used to confirm PCR test results. The HI assay is unsuitable for detecting non-psittacine raised antibodies due to the presence of non-specific factors that may be present in the serum and cause false-positive reactions. BFDV-specific antibodies were successfully raised in rabbits in this study. The ELISA can be standardised and used as an additional serologic diagnostic test to the HA and HI assays.

CHAPTER 7.

SUMMARY.

Psittacine beak and feather disease (PBFD) is a readily recognizable disease of wild and captive psittacines in Australia but it is also a problem worldwide wherever captive species are bred. The disease caused by beak and feather disease virus (BFDV) is characterized by the progressive development of feather dystrophy and loss. Although the occurrence of PBFD in South Africa has been reported only recently, it is already rampant and threatens the extinction of the endangered Cape parrot and black-cheeked lovebird.

Currently no vaccine for PBFD is commercially available but the loss of approximately 10-20% of breeding stocks of psittacines annually in South Africa alone is enough to realise the importance of one. Genetic and antigenic differences in BFDV are significant aspects for production of a vaccine but the lack of a culture system for BFDV has limited studies into its genetics, antigenicity and pathogenicity. The objective of the study thus became to establish techniques that could be used to investigate genetic and antigenic differences that may be present in BFDV in South African psittacines.

Molecular investigations involved the testing dried blood samples for BFDV nucleic acid using polymerase chain reaction (PCR). A region within the Rep gene was amplified and digested with *Hae*III to yield restriction length fragment polymorphisms (RFLPs). Six RFLPs were identified, cloned, sequenced (UFS 1-6) and phylogenetically analysed. BFDV was purified from body organs of PBFD-affected birds by cesium chloride density gradient centrifugation and fractions tested by PCR and haemagglutination (HA) assays. BFDV-specific antibodies were raised in two rabbits by inoculation with purified BFDV in Freund's incomplete adjuvant and tested by haemagglutination inhibition (HI) assays and an enzyme-linked immunosorbent assay (ELISA). HA and HI assays were

attempted using erythrocytes from African grey parrots and Brown-headed parrots. HI assays were also used to test parrot sera for the presence of antibodies to BFDV.

UFS 1-6 were closely related to known BFDV isolates and UFS 1 may represent a unique genotype in South Africa as it was separated from its closely related isolates by a 90% bootstrap value. UFS 3, 4 and 5 isolated from budgerigars belong to the budgerigar lineage as they clustered with isolate BG3-NZ. Together with three previously identified genotypes, UFS 1 indicates the introduction of BFDV into southern Africa on four separate occasions.

Purification of BFDV from organs was successful but yielded low titres possibly because low quantities of virus were present in the organs or because little virus was circulating in the bird upon its death. PCR and HA assays confirmed the presence of BFDV in fractions; the two results correlated well.

HA and HI assays were successfully established using erythrocytes from African grey parrots and Brown-headed parrots. Antibodies to BFDV were successfully detected in sera of three parrots by the HI assay. However, the assay could not detect non-psittacine raised antibodies (rabbit-raised) due to non-specific reactions. BFDV-specific antibodies were successfully raised in rabbits and were verified by the use of an ELISA.

The high level of genetic diversity observed in the study compels further investigation into the genetics of BFDV as such levels of diversity may become a limiting factor in the applicability of PCR as a diagnostic test. The entire diversity of BFDV has not been studied and future work may lead to the identification of more BFDV strains, an important factor in vaccine development. The rabbit-raised antibodies together with the HA and HI assays and ELISA can be used to study the antigenic differences that may be present in known BFDV isolates that may also lead to the identification of more strains of the virus.

OPSOMMING.

Papegaai bek en veer siekte (PBVS) is 'n geredelike herkenbare siekte van wilde en gehokte voëls in Australië maar, is ook 'n wêreldwye probleem waar ookal met voëls geboer word. Die siekte word veroorsaak deur die bek en veer siekte virus (BVSV) en word gekarateriseer deur erge veer distrofie en verlies. Alhoewel die voorkoms van PBVS slegs onlangs in Suid Afrika geraporteer is, versprei dit vinnig en bedreig dit die bestaan van die Kaapse papegaai en die black-cheeked lovebird.

Daar is tans geen kommersiël besikbaar entstof teen die virus nie en die belang van so 'n entstof word beklemtoon deur die verlies van 10-20% broeivoëls jaarliks in Suid Afrika alleen. Die tekort aan 'n kwekings sisteem vir BVSV het beperkings geplaas op studies van die genetika, antigenisiteit en patogenisiteit van die virus. Verskille in genetika en antigenisiteit van die BVSV is belangrike aspekte in die ontwikkeling van 'n entstof.

Die doel van die studie was die ontwikkeling van tegnieke wat gebruik kan word om genetiese en antigeniese verskille te ondersoek wat voorkom in Suid Afrikaanse BVSV isolate.

Die teenwoordigheid van BVSV nukleïen sure in gedroogde bloedmonsters was bevestig deur die polymerase ketting reaksie (PKR). 'n Gedeelte binne die Rep-geen was geamplifiseer en verteer deur die ensiem *Hae*III om beperkings lengte fragment polimorfismes (BLFPs) te verkry. Ses BLFPs was geidentifiseer, gekloneer, die basis paar opeenvolging bepaal (UFS 1-6) en filogeneties geanaliseer. BVSV was gesuiwer deur middle van cesium chloride digtheid gradient sentrifugering uit liggaamsorgane verkry vanaf geinfekteerde papegaaie. Die gesuiwerde fraksies was deur PKR en hemagglutinasie (HA) getoets. BVSV spesifieke teenliggaampies was gekweek in hase deur inokulasie met gesuiwerde virus in Freund se onvolledige adjuvant. Die gekweede

teenliggaampies was deur hemagglutinasie inhibisie (HI) en enzyme linked immunosorbent assay (ELISA) getoets. Die HA en HI toetse was ontwikkel deur rooi bloed selle (RBS) van African grey en Bruin-kop papegaai te gebruik. Die teenwoordigheid van BVSV teenliggaampies in papegaai sera was ook bevestig deur HI toetse.

UFS 1-6 was na-verwant aan bekende BVSV isolate. UFS 1 verteenwoordig 'n unieke genotipe van Suid Afrika en was geskei van ander isolate deur 'n bootstrap waarde van 90%. UFS 3, 4, 5 en BG3-NZ was geisoleer uit grasparkiete en die virus isolate behoort aan die grasparkiet linie. Saam met drie vorige geidentifiseerde genotipes, dui UFS 1 aan dat BVSV op vier verskillende geleenthede suiderlike Afrika besmet het.

BVSV was suksesvol gesuiwer uit liggaamsorgane maar het lae titres gelewer en dit is waarskynlik as gevolg van die lae konsentrasie virus in papegaaie op die tyd van dood. PKR en HA toetse het die teenwoordigheid van BVSV in die fraksies bevestig en die resultate korreleer goed.

Suksesvolle HA en HI toetse was ontwikkel deur gebruik te maak van RBS van African grey en Bruin-kop papegaaie. Die teenwoordigheid van BVSV teenliggaampies in sera van drie papegaaie was bevestig deur HI toetse. BVSV teenliggaampies was suksesvol gekweek in hase. Die teenwoordigheid kon egter nie bevestig word deur HI toetse nie maar wel deur ELISA toetse.

Die hoë vlak van genetiese diversiteit waargeneem tydens die studie noodsaak verdere navorsing van die genetika van BVSV. Die vlakke van diversiteit kan 'n beperkende faktor word in die aanwendbaarheid van PKR as diagnostiese toets. Die algehele diversiteit van die BVSV is nog nie bestudeer nie. Toekomstige studies mag vele meer BVSV stamme ontdek en dit is 'n baie belangrike faktor in entstof ontwikkeling. Antigeniese verskille wat mag voorkom in bekende BVSV isolate kan deur die haas gekweekde teenliggaampies. HA, HI en ELISA toetse

bevestig word, wat mag lei tot die ontdekking van nog meer stamme van die virus.

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APPENDIX A.

REAGENT	SUPPLIER
λ phage DNA	MBI Fermentas
50bp DNA Step Ladder	Promega
Agarose D1 LE	Whitehead Scientific (PTY) LTD
Anti-Rabbit IgG	Sigma®
Beckman Quickseal Ultraclear™ Centrifuge tubes	Beckman
$CaCl_2$	Merck
Citric acid	Merck
Costar ELISA plates	Sterilab Services
CsCl	Sigma®
*Eco*RI	Roche
EDTA	Merck
Ethanol (99.5%)	Merck
Freund's incomplete adjuvant	Sigma®
GFX™ PCR DNA and Gel Band Purification Kit	Amersham Biosciences
GFX™ Microplasmid Prep Kit	Amersham Biosciences
Glacial acetic acid	Merck
Glucose	Merck
Glutaraldehyde (25%)	Sigma®
Greiner 96 well V-bottom microtiter plates	Laboratory and Scientific Equipment (PTY) LTD
*Hae*III	Roche
HCl (32%)	Merck
*Hind*III	Roche
H_2O_2	Merck
H_3PO_4	Merck

APPENDIX A.

REAGENT	SUPPLIER
H_2SO_4	Merck
Isopropanol	Merck
JM 109 *Escherichia coli* competent cells	Promega
KCl	Merck
KH_2PO_4	Merck
KOH pellets	Merck
Low melt Agarose # LM Sieve	Whitehead Scientific (PTY) LTD
$MgCl_2.6H_2O$	Merck
NaCl	Merck
Na_2CO_3	Merck
$NaHCO_3$	Merck
NaH_2PO_4	Merck
Na_2HPO_4	Merck
NaOH pellets	Merck
O'GeneRuler™ DNA Ladder	MBI Fermentas
Oligonucleotide primer set PBF F1 and PBF R1	Southern Cross Biotechnology (PTY) LTD [QIAGEN Operon]
PCR reaction buffer 10X concentration	Roche
PEG	Merck
pGEM™ TEasy Vector System I	Promega
QIAamp® DNA Mini Kit	Southern Cross Biotechnology (PTY) LTD [QIAGEN]
SDS	Merck
Set of dATP, dCTP, dGTP and dTTP	Roche
Sigmaspin™ Post Reaction Purification Columns	Sigma®

APPENDIX A.

REAGENT	SUPPLIER
Slide-A-Lyzer® Dialysis Cassette	Pierce
SuperTherm DNA Polymerase	Southern Cross Biotechnology (PTY) LTD
T4 DNA ligase	MBI Fermentas
TMB	Sigma®
Tris	Merck
Trisodium citrate	Merck
Tryptone powder	Merck
Tween 20	Sigma®
Whatman number 1 paper	Separations
Yeast extract powder	Merck

APPENDIX B.

1. <u>ALSEVER'S SOLUTION.</u>

Dextrose	20.50 g
Trisodium citrate	10.88 g
Citric acid	0.55 g
NaCl	4.22 g

The final volume was made up to one liter with distilled water and sterilized by autoclaving.

2. <u>CARBONATE BUFFER (50 mM, pH 9.6)</u>.

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

800 ml of distilled water was added and the pH adjusted to 9.6 using concentrated HCl. The final volume was made up to one liter.

3. <u>ELISA BLOCKING BUFFER (5% w/v)</u>.

ELITE milk powder	5.00 g
TST buffer	100 ml

4. <u>GA-SALTS SOLUTION (1% v/v)</u>.

25% Glutaraldehyde stock solution	1 volume
Salts solution	24 volumes

APPENDIX B.

5. <u>GLUCOSE-TRIS-EDTA [GTE].</u>

    2 M Glucose   1.25 ml

    Tris-HCl   1.25 ml

    0.5 M EDTA   1.00 ml

Made up to 50 ml with distilled water.

6. <u>0.2 M NaOH/ 1% SDS.</u>

    10 M NaOH   1 ml

    10% w/v SDS   5 ml

Made up to 50 ml with distilled water.

7. <u>PHOSPHATE BUFFERED SALINE [PBS].</u>

    0.2 M NaH_2PO_4 19 ml

    0.2 M Na_2HPO_4 81 ml

    NaCl   1.7 g

Made up to 200 ml with distilled water.

APPENDIX B.

8. <u>PBS – Ca^{2+}, Mg^{2+} (FOR CONCENTRATION OF RBCs).</u>

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Dissolved in one liter of distilled water in the order listed and sterilized by autoclaving.

9. <u>PBS + Ca^{2+}, Mg^{2+} (FOR HA AND HI ASSAYS).</u>

Solution A.

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water	800 ml

Solution B.

$CaCl_2$	0.1 g
$MgCl_2.6H_2O$	0.1 g
Distilled water	200 ml

The salts were dissolved in the distilled water in the order listed and sterilized separately by autoclaving. Solutions A and B were mixed aseptically when cooled.

APPENDIX B.

10. <u>POTASSIUM ACETATE [KAc] (5 m, pH 4.8).</u>

    Glacial acetic acid   29.50 ml

    KOH

    The pH was adjusted to 4.8 using KOH and made up to 100 ml with distilled water.

11. <u>SALTS SOLUTION.</u>

    0.15 M Na_3PO_4* (pH 8.2)   1 volume

    0.15 M NaCl   24 volumes

    Distilled water   5 volumes

    *Prepared as 0.15 M H_3PO4 adjusted to pH 8.2 by the addition of NaOH pellets.

12. <u>TRIS-EDTA [TE] (10mM TRIS, 1mM EDTA, pH 8.0).</u>

    1 M Tris-HCl (pH 8.0)   2 ml

    0.5 M EDTA (pH 8.0)   400 µl

13. <u>4,4,5,5'-TETRAMETHLYBENZIDINE [TMB] (SUBSTRATE FOR ELISA).</u>

    TMB tablet   1

    0.1 M Citric acid   5.5 ml

    0.2 M NaH_2PO_4 5.5 ml

    H_2O_2 10 µl

APPENDIX B.

14. <u>TRIS (5 mM, pH 8.0).</u>

    Tris   0.61 g

Dissolved in 800 ml of distilled water and adjusted to pH 8.0 with concentrated HCl. The final volume was made up to one liter with distilled water.

15. <u>TRIS-SODIUM CHLORIDE-TWEEN 20 [TST] (1X, pH 8.0).</u>

    Tris   7.88 g

    NaCl   8.77 g

    Tween 20   1.0 ml

The pH was adjusted to 8.0 with 32% HCl and the volume made up to one liter.