

Antigenic investigation of genetically different strains of *Beak and feather disease virus*

by

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*For my late grandfather, Han Gouweloos, who always inspired me to
know more...*

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CHAPTER 1: PSITTACINE BEAK AND FEATHER DISEASE: LITERATURE REVIEW

1.1. INTRODUCTION

Psittacine beak and feather disease (Pbfd) is a dermatological condition in parrots caused by *Beak and feather disease virus* (BFDV) (Shoemaker *et al.*, 2000) and was first recognized and described in 1975 by Dr. Ross Perry in Sydney, Australia. Since then Pbfd is recognized as the most common disease of wild and captive Old World Psittacines as well as New World Psittaciformes.

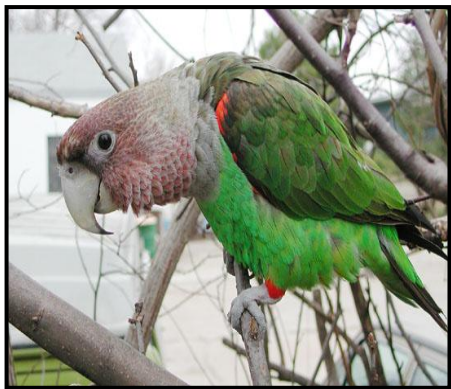
Originally Pbfd was only found in wild psittacines in Australia, but due to the worldwide trade in psittacine species, the disease is not limited to Australian psittacines, but affects species of every continent (de Kloet and de Kloet, 2004). According to the Australian Commonwealth Government, Pbfd is listed as a key threatening process to the survival of five endangered species. One of these critically endangered species is the Orange-bellied parrot (*Neophema chrysogaster*) of which there are about 180 breeding birds remaining

(www.environment.nsw.gov.au/determinations/beakandfeatherdiseasektplisting.htm).



Figure 1.1: The Orange-bellied parrot which is one of the psittacine birds on the red species list in Australia (<http://www.arkive.org/orange-bellied-parrot/neophema-chrysogaster/>).

In Africa the disease threatens the survival of the indigenous endangered Cape parrot (*Poicephalus robustus*) and the black-cheeked lovebird (*Agapornis nigrigenis*) (Heath *et al.*, 2004).



A



B

Figure 1.2: A Cape parrot (A) (www.wishes.debian.co.nz) and black-cheeked lovebirds (B) (http://www.strayreality.com/birding_directory_site/birding/lovebirds02.jpg) which are endangered psittacines in Africa.

PBFD is caused by a circovirus belonging to a diverse group of plant and animal pathogens which have undefined relationships to one another, except that they all share

non-enveloped capsids with circular, single stranded genomes (Niagro *et al.*,1998). Related viruses have been identified and characterized in domestic pigeons (*Pigeon circovirus*, PiCV), common canary (*Canary circovirus*, CaCV), domestic geese (*Goose circovirus*, GoCV), ducks (*Duck circovirus*, DuCV) and in pigs (*Porcine circovirus*, PCV) (de Kloet and de Kloet, 2004).

BFDV occurs only in psittacine species, affecting over 40 different species (Ritchie *et al.*, 1992a). Very little is known about BFDV isolates found outside Australia. This disease is a major problem for bird breeders in South Africa, where about 10 – 20% of South African psittacine breeding stocks are lost due to the disease each year (Heath *et al.*, 2004).

1.2. PSITTACINE BEAK AND FEATHER DISEASE

1.2.1. THE CLINICAL ASPECTS OF Pbfd

Pbfd occurs mainly in captive young birds which are younger than three years old. Affected birds loose contour feathers over most of their bodies; the feather loss pattern is variable and is dependent on the stage of moult the bird is in during onset of the disease. Feather loss is roughly symmetrical with normal plumage gradually replaced by abnormal feathers with the following characteristics; retained feather sheath, blood in the feather shaft, short clubbed feathers, curled and deformed feathers, feathers with circumferential constrictions and stress lines in the vane. Lesions also occur in the major tail feathers (Pass and Perry, 1984).

The upper and lower beaks develop progressive changes in colour and growth (Pass and Perry, 1984); with the upper beak being more affected than the lower beak (Ritchie and Carter, 1995). The beak changes from a semi-gloss to gloss black colour, with progressive elongation, development of fault lines, breakage and under running of the outer and oral surface (Pass and Perry, 1984). Uneven wear, chips and bacterial infection contribute to impaired ability to eat and can lead to further debilitation and

weight loss (Jergens *et al.*, 1988). Beak pathology is not always present in BFDV infected birds and seems to be dependent on the species involved (Ritchie *et al.*, 1989). These beak abnormalities occur more commonly in larger species such as sulphur-crested cockatoos, galahs and corellas. Similar changes may occur in the claws and sometimes the claws may slough (Pass and Perry, 1984). Some birds die soon after showing the first clinical symptoms such as malformed feathers, where others live for years in a featherless state (Ritchie *et al.*, 1989).

BFDV affects the organs of its host, including feathers, liver, brain and the immune system (de Kloet and de Kloet, 2004). The incubation period of BFDV is approximately 21 days, but is dependent on the dose of virus, age of the bird, the stage of feather development the bird is in and the absence of immunity in birds (<http://numbat.murdoch.edu.au/caf/BFDV.htm>).

Types of clinical disease varies and is controlled by the age of the bird, the route of viral exposure, titer of infecting virus and the condition of the bird during exposure (Ritchie and Carter, 1995):

- I. The acute form of this disease is commonly observed in young birds during formation of the first feathers after the replacement of the neonatal down. The infections can be characterized by necrosis, fracture, bending, haemorrhaging or the premature shedding of the developing feathers. Chicks which develop clinical lesions while the feathers are still in developmental stage exhibit the most severe feather pathology. Progression of the disease is less dramatic in young birds that develop clinical symptoms after body contour feathers are mature (Dahlhausen and Radabaugh, 1998).
- II. In peracute cases in young birds (soon after hatching) the disease is characterized by depression, anorexia, enteritis (vomiting and diarrhoea), septicaemia, pneumonia and rapid death within 1 – 2 weeks. No feather abnormalities will have been observed in these birds as they will still be covered

in neonatal down (Dahlhausen and Radabaugh, 1998; Shoemaker *et al.*, 2000; Todd *et al.*, 2001).

- III. A chronic form of the disease is observed in older birds where dystrophic feathers, which stop growing after emerging from the follicle, appear during each successive moult (Dahlhausen and Radabaugh, 1998). A change in colour of the feathers may also be observed; feather loss appears in a roughly symmetrical pattern and is then replaced by the dystrophic feathers (Todd, 2000). Powdery down feathers on the flank region are the first to show signs of disease. The disease progresses to affect the contour feathers in most feather tracts and is followed by dystrophic changes in the primary and secondary feathers of the wings, tail and crest (Dahlhausen and Radabaugh, 1998). Baldness results when the feather follicles become inactive. Beak and nail tissues may also be affected, causing deformities, especially in Cockatoos (Todd, 2000).

The disease is considered fatal, with most infected birds surviving between six months and two years after onset of clinical signs (Dahlhausen and Radabaugh, 1998).



A



B

Figure 1.3: An Eclectus (A) (Bendheim *et al.*, 2006) and an African Grey (B) (<http://www.theaviary.com>) showing signs of feather loss and feather dystrophy.

1.2.2. PATHOLOGY

BFDV is suggested to be epitheliotropic in feathers and follicles - targeting replicating cells within the basal layers of the epithelium (Latimer *et al.*, 1991).

Clinical abnormalities of the feathers, beak and claws are due to a combination of dystrophy and hyperplasia in the epidermis of the feather follicles, beak and claws. The dystrophy is due to necrosis (which according to Trinkaus *et al.*, 1998, is the consequence of secondary infections due to immunodeficiency and is not primarily associated with the virus-induced mechanism of pathogenicity) and hyperplasia of epidermal cells. Hyperplasia produces hyperkeratosis of the feather sheath and outer layers of the beak and claws. The dystrophy produces short and abnormally shaped feathers (Pass and Perry, 1984). The circumferential indentations that are seen in

many feathers are the result of thinning of the epidermis of the rachis and may be due to a period of slow growth or excessive necrosis.

Beak overgrowth is due to hyperkeratosis and the failure of keratinized layers to slough, degenerative changes in the epidermis lead to splitting of the epidermal layers which can become infected with bacteria (Todd, 2000; Latimer *et al.*, 1991).

Feather pulp lesions are characterized by inflammation that involves infiltration of heterophils, plasma cells, macrophages and lymphocytes (less common). The bursa of Fabricius and thymus of affected birds show lesions consisting of atrophied lymphoid tissue and aggregates of necrotic tissue. Severe bursal and thymic necrosis may be the only histological lesions in birds suffering from the peracute form of the disease (Todd, 2000).

PBFD occurs with cell death which morphologically resembles apoptosis and these apoptotic bodies serve as a vehicle for dissemination of the viral particles (Trinkaus *et al.*, 1998).

1.2.3. IMMUNOSUPPRESSION

PBFD is commonly associated with immunodeficiency-related diseases which are caused by the depletion of lymphoid tissue with damage to the lymphoreticular tissue being pronounced in the bursa of Fabricius and the thymus (Latimer *et al.*, 1990, Heath *et al.*, 2004). The viruses are thought to target precursor T cells depleting populations of both helper (CD⁴⁺) and cytotoxic T (CD⁸⁺) cells (Ritchie *et al.*, 2003). Death occurs due to secondary viral, bacterial or fungal infections. Complications that arise from terminal disease necessitate euthanasia (Dahlhausen and Radabaugh, 1998).

With mature, functioning immune systems, most birds are capable of mounting an effective and protective immune response, which can result in elimination of the virus, thus the bird is “naturally vaccinated”. The maturity of the immune system in relation to

the time of viral exposure is a determining factor in progression of the disease in baby birds (Dahlhausen and Radabaugh, 1998).

1.2.4. TRANSMISSION

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 transmits viral particles during preening and feeding activities. Low concentrations of BFDV are found in the crops of infected birds, that leads to transmission of the virus to neonates during feeding – which involves regurgitation of food. The viral source could be attributed to infected cells in the crop or oesophageal epithelium or swallowed deposits of exfoliated epithelium from beak or oral mucosal lesions (Ritchie *et al.*, 1991a).

Feather dust is indicated to be a major vehicle of transmission as a high concentration of virus is found in feather dust and it can be dispersed with ease through air flow and contact with contaminated clothing, nets, bird carriers, food and insects (Ritchie and Carter, 1995).

Another mode of transmission was suggested after the recovery of BFDV from faeces. Nestling psittacine birds sit tripod-like on their legs and abdomen until they have developed a sense of balance. During defaecation they rub their cloaca over the nesting material, which allows BFDV particles to gain access to the bursa of Fabricius by direct cloacal infection (Raidal *et al.*, 1993).

According to Maramorosch *et al.* (2001), vertical transmission was indicated when artificially incubated chicks from BFDV infected hens consistently developed PBFD. Rahaus and co-workers (2008) supported the occurrence of vertical transmission in a study where BFDV deoxyribonucleic acid (DNA) was present in embryonated and non-embryonated eggs. Several reports indicate that asymptotically infected adult

birds can produce clinically infected young in successive breeding seasons that suggest the existence of a carrier state from which horizontal or vertical transmission of BFDV may occur.

1.2.5. DIAGNOSIS

PBFD is difficult to diagnose on the basis of clinical features alone as there are many causes of feathering problems in birds. Difficulty in diagnosis also occurs when birds show only subtle signs of the disease due to age or the state of their immune system and when the virus is incubating and no symptoms are present (<http://numbat.murdoch.edu.au/caf/BFDV.htm>).

The diagnoses of PBFD is based upon clinical symptoms, such as the presence of histological lesions in affected feathers as well as the demonstration of viral particles in feather homogenates and smears (Wylie and Pass, 1987). PBFD should be considered in any psittacine bird that displays progressive feather loss or abnormal feathers (www.theaviary.com). Test results should be confirmed by the Polymerase Chain Reaction (PCR) to eliminate other causes of feather loss such as *Avian polyoma virus* (APV) and bacterial infection of the feathers.

The virus can not be cultivated in tissue/cell culture or in embryonated eggs; thus routine diagnosis of the disease and the development of diagnostic tests are restricted (Johne *et al.*, 2004). Diagnosis of the infection must be confirmed by demonstration of viral antigen or viral nucleic acid (Todd, 2000).

Several methods for the detection of BFDV infection have been developed. At first, diagnosis of PBFD was mainly performed by histological examination of feather follicles to confirm clinical disease, but is not suitable for incubating infections. In comparison with skin biopsies, the examination of feather follicles is more rapid, economical and non-invasive as it requires examination of plucked feathers with the feather epithelium and epidermal collar intact (Latimer *et al.*, 1991). BFDV produces basophilic

intracytoplasmic or intranuclear inclusion bodies in the feather pulp, feather follicle skin or the bursa of Fabricius. However, the absence of inclusion bodies on histopathology does not exclude Pbfd (Pyne, 2005), but in the presence of inclusion bodies a confirmatory diagnosis (detection of viral-specific antibodies or detection of viral DNA) is required due to the similarity of inclusion bodies caused by APV and Adenoviruses. Immunohistochemical staining with rabbit anti-BFDV antibodies has been used for the confirmation of inclusion bodies in haematoxylin and eosine stained tissue sections that contain BFDV antigen (Ritchie *et al.*, 1992b).

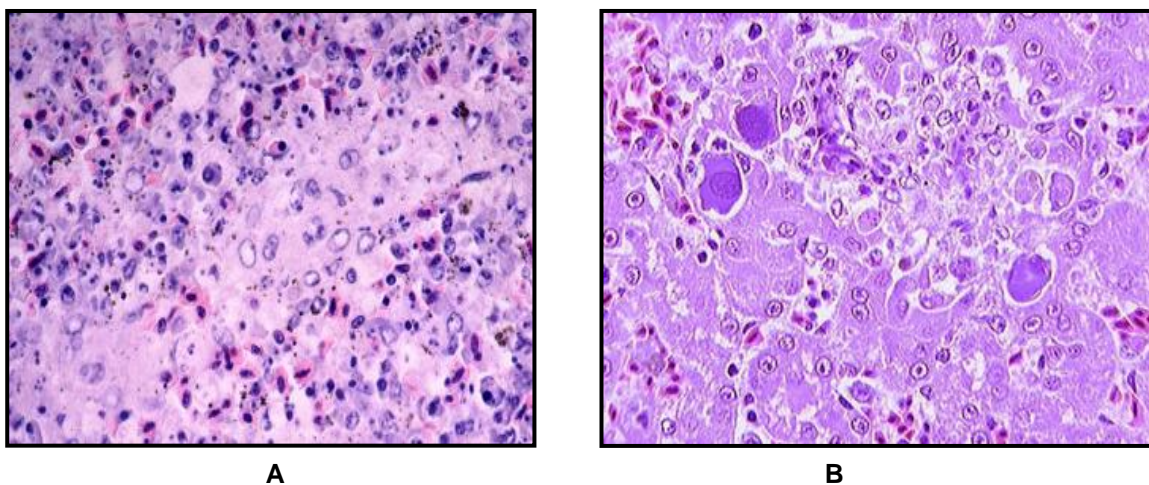


Figure 1.4: Histological examination of tissue samples depicting the similarity of APV (A) and BFDV (B) inclusion bodies, (www.theparrotsocietyuk.com).

Diagnosis can be done by finding viral antigen with the use of a DNA probe which detects BFDV nucleic acid in the white blood cells of infected birds (Ritchie *et al.*, 1992a). BFDV and APV specific DNA probes were used by Latimer *et al.* (1993) to rapidly and economically confirm or exclude concurrent BFDV and APV infections in birds, *in situ* hybridization is less sensitive than DNA probes, but in conjunction they form a better diagnostic test.

An indirect enzyme linked immunosorbent assay (ELISA) and immunoblotting was used by Johne and co-workers (2004) who cloned part of the region which encodes the capsid protein C1 and applied a polyhistidine-tailed variant of this protein as a

recombinant antigen to test for BFDV-specific antibodies. Individual isolates of BFDV differ within the C1 gene, which limits the application of the use of recombinant antigen in serological tests.

Currently the most widely used serological test to detect BFDV antigen and antibodies is the haemagglutination (HA) and haemagglutination inhibition (HI) assays respectively, as an optimized ELISA has not been established (Kondiah, 2004). Virus can be detected in affected feathers with the use of HA and antibodies can be detected in the blood, serum, plasma or yolk using the HI test. The HA test is used to determine the level of virus in a bird and the HI will assess the immune response of a bird. Combined results give an accurate picture of the outcome of the disease (Pyne, 2005). 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 be used to determine the seroprevalence of BFDV antibodies in captive and wild psittacine birds (Ritchie *et al.*, 1991b). Stewart and co-workers (2007) applied recombinantly expressed coat protein (CP) in a HA test in order to determine the minimum concentration of CP needed to cause HA, they found that HA was detected at concentrations between 15.2 – 21.2 nanograms (ng), no HA occurred at dilutions below 14.0 ng or less.

Although useful, it is difficult to choose suitable erythrocytes for HA assays because the HA activity of BFDV differs for erythrocytes of different species and also amongst individuals of the same species (Sanada and Sanada 2000). *Cacatua galerita* (sulphur crested cockatoo) erythrocytes have been described as the most sensitive for detection. Another drawback of HA assays is that it doesn't detect latent or incubating BFDV infection and also the possible genetic or antigenic diversity of BFDV limits the applicability of this test (Raidal *et al.*, 1993; Johne *et al.*, 2004). The ability of BFDV to agglutinate erythrocytes is unaffected by temperature, indicating stability of the virus (Todd, 2000).

In South Africa, the HA and HI assays are unavailable as a diagnostic test because most of the psittacine species are not readily available as they are not indigenous to

Africa. In addition the birds are expensive to acquire and not all individuals of a species are suitable sources of erythrocytes for the BFDV HA assay. However, according to a study done by Kondiah (2004), African Grey parrots and Brown Headed parrots provide a local and economical source of red blood cells for application in the HA and HI assays to detect BFDV antigen and antibodies, respectively, in South Africa.

PCR-based techniques are used for detecting virus DNA in feather follicle material from clinically infected birds, in blood of asymptomatic birds and in swabbed material collected from cages and enclosures (Schoemaker *et al.*, 2000). PCR is probably the most sensitive test for detecting latent or incubating BFDV infection, but according to Riddoch and co-workers (1996) the results are not quantitative.

A universal PCR test was developed by Ypelaar *et al.* (1999), based on the assumption that there was only one strain of BFDV worldwide that consistently detects BFDV infections in a diversity of psittacine birds. The PCR amplifies a 717 base pair (bp) fragment of ORF1 which contains the code for three of the four motifs involved in rolling circle replication (RCR) (Ypelaar *et al.*, 1999).

A negative PCR result is a strong indication that a bird is not infected, but should be interpreted in conjunction with the clinical signs, age of the bird and circulating antibody titers (Kondiah, 2004). Birds which test positive for BFDV infection, if they do not show clinical symptoms, need to be retested again in 60 – 90 days. It is possible for a bird to undergo transient sub-clinical infection, meaning that the bird's immune system is able to eliminate the virus, which is why a normal appearing bird that tests positive should be retested 90 days later. If the bird has eliminated the virus it will test negative, but if it remains positive, the bird can be considered latently infected.

Recently a monoclonal antibody to a recombinant BFDV CP has been developed and its use in Western blotting, immunohistochemistry, ELISA and HI's were evaluated. The antibody was found to be specific for both recombinant BFDV CP and the whole virus; similar optimal titers were found when this antibody was used in Western blotting

and immunohistochemistry. The monoclonal antibody also had HI activity and detected BFDV from three genera of birds (Shearer *et al.*, 2007). This use of monoclonal antibodies against recombinant protein is promising in the development of reliable assays for the diagnosis of PBFD in birds.

1.2.6. TREATMENT

Treatment of diseased birds is at best, supportive only. Due to extensive feather loss, thermoregulation in diseased birds is impaired and they should be housed in warm, draft-free environments. A balanced diet, along with antibiotics to combat secondary bacterial, fungal and parasitic infections should be provided (Jergens *et al.*, 1988).

It has been found with HI that birds with active PBFD infections had lower anti-PBFD virus antibody titers than birds exposed to the virus which remained clinically normal – this suggests that some birds exposed to the virus are able to mount an effective immune response (Ritchie *et al.*, 1992a). Spontaneous recovery from acute BFDV infections can occur in many species, including budgerigars, lorikeets and lovebirds, but the majority of chronically affected birds do not recover from the disease (Jergens *et al.*, 1988, <http://numbat.murdoch.edu.au/caf/BFDV.htm>).

1.2.7. PREVENTION AND CONTROL

An outbreak of PBFD is difficult to contain as the virus is resistant to many control measures and environmental degradation (Todd, 2000; Ritchie *et al.*, 2003). The disease is also difficult to quarantine, as carrier birds appear normal and lack symptoms. Carrier birds also produce diseased young through vertical transmission. This necessitates for the breeding of birds to occur in quarantine to prevent further spread of the disease. Aviculturists are advised to maintain closed flock and to only purchase birds from PBFD-free flocks (<http://numbat.murdoch.edu.au/caf/BFDV.htm>).

The time in which BFDV remains viable in the environment has not been established, but it is accepted that BFDV is viable for a long period of time. Thorough cleaning of

nursery premises has eliminated the problem of paediatric infection (Dahlhausen and Radabaugh, 1998). Neonates who are most susceptible to BFDV infection must not be exposed to areas that are contaminated by faeces or feather dust from PBF positive birds. The repeated use of disinfectants (e.g. gluteraldehyde which inactivate environmentally resistant viruses) is recommended for disinfecting contaminated cages, utensils and rooms to remove any residual virus shed by the birds. The efficacy of the product Virukill[®] Avian has been tested against *Chicken anemia virus* (CAV) and this product has been demonstrated to inactivate this virus (Bragg 2008, personal communication). As CAV is also a circovirus, it is suggested that Virukill[®] Avian will inactivate BFDV. Separate air flow systems in examination and treatment areas will prevent the spread of the virus by air.

A vaccine consisting of inactivated BFDV in a double-oil emulsion adjuvant system has been investigated by Raidal *et al.* (1993). Its use can be a safe and effective aid for controlling the disease if it is combined with other management procedures, such as biosecurity (Albertyn *et al.*, 2004). The vaccine is not a treatment for already infected birds. If administered to diseased birds it can exacerbate the disease process. Birds should be vaccinated at a very young age (as young as 14 days) and a booster vaccination should be administered one month after the first vaccination and afterward birds should be examined every six months until they are three years of age. Breeding birds should be vaccinated one month prior to breeding, so the antibodies can be transmitted to the young (<http://numbat.murdoch.edu.au/caf/BFDV.htm>). The vaccine is not commercially available as not all species are protected by the inactivated vaccine and protection varies when administered to older birds, thus it cannot be used specifically as a PBF preventive measure. Although the vaccine has been developed, the inability to propagate BFDV *in vitro* in tissue/cell cultures has hampered the use of an attenuated or killed vaccine for commercial use in the prevention of PBF as it is difficult to obtain enough virus with which to manufacture these vaccines.

At present the development of a vaccine that will effectively protect parrots against Pbfd relies on molecular biology techniques, such as the development of sub-unit or DNA vaccines.

1.3. BEAK AND FEATHER DISEASE VIRUS

1.3.1. TAXONOMY

The 8th report of the International Committee on Taxonomy of Viruses (ICTV) classified *Beak and feather disease virus*, *Canary circovirus*, *Goose circovirus*, *Pigeon circovirus* and *Porcine circovirus* into the genus *Circovirus* within the family *Circoviridae*. *Chicken anemia virus* (CAV) has been reclassified into the genus *Gyrovirus*, but remains within the family *Circoviridae*. Virions in the *Circoviridae* are non-enveloped, icosahedrons, 17 – 22 nanometers (nm) in diameter with covalently closed, circular, negative-sense, single-stranded DNA (ss-DNA) genomes between 1.7 – 2.3 kilobases (kb) in size, representing the smallest viral DNA replicons known (Niagro *et al.*, 1998).

1.3.2. MORPHOLOGY

BFDV has a non-enveloped, icosahedral or spherical capsid with no obvious surface structures. The virus has a diameter of between 14 and 17 nm, which makes it one of the smallest animal viruses (Maramorosch *et al.*, 2001).

According to Ramis *et al.* (1998), intranuclear and intracytoplasmic basophilic inclusions have been identified in follicular epithelial cells and in macrophages of the feather pulp respectively. Other tissues where virus 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 the adrenal glands.

With the use of electron microscopy, the intracytoplasmic inclusions have been reported to consist of electron dense granules 17 – 22 nm in diameter (Pass and Perry, 1984)

that form paracrystalline arrays, semicircles, concentric circles and whorls etc. (Trinkaus *et al.*,1998).

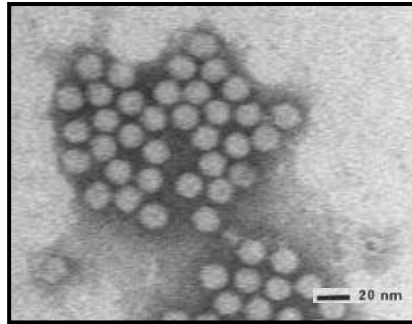


Figure 1.5: Electron Micrograph of negatively stained *Beak and feather disease virus* particles (<http://numbat.murdoch.edu.au/caf/BFDV.htm>).

1.3.3. GENOME

The family *Circoviridae* consists of the animal pathogens (BFDV and PCV) that possess ambisense genomes with similarities to the geminiviruses (Niagro *et al.*, 1998). The genome of BFDV possesses a circular, ss-DNA molecule of between 1992 – 2018 nucleotides (nts) (Todd, 2000).

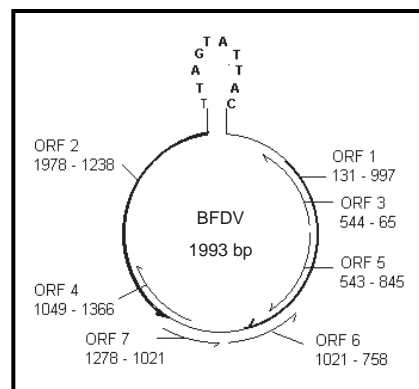


Figure 1.6: The circular ss-DNA genome of BFDV showing the conserved nonanucleotide motif (TAGTATTAC) and the seven ORFs (Bassami *et al.*, 1998).

BFDV contains seven major ORFs which encode proteins >8.7 kiloDaltons (kDa). Three of these ORFs are on the virus sense strand and four ORFs on the

complementary sense strand of the replicative form (RF). Not all the ORFs are present in all isolates, but ORFs 1, 2 and 5 are conserved among all isolates. The genome lacks a distinct non-coding region (Bassami *et al.*, 1998) and it contains two major ORFs in opposite orientation, which encode the replication associated protein (Rep, ORF1) and the coat protein (CP, ORF2) (Niagro *et al.*, 1998). A third ORF (ORF5) – which is common to all BFDV isolates – has been described, but it is unclear what role its transcriptional product plays in replication of the virus, the ORFs described by Bassami and co-workers (1998) are putative, there is no evidence that any ORFs are actively transcribed other than the CP and Rep. The start codons for these ORFs were in all cases ATG, except for ORF2 where ATG, CTG or TCT have been shown to be start codons (Bassami *et al.*, 2001).

The BFDV genome contains a potential stem-loop structure, which at its apex contains a conserved nonanucleotide motif (TAGTATTAC) between the start sites of ORF1 and ORF2 (Todd *et al.*, 2001), which is also conserved among plant geminiviruses, plant nanoviruses and bacteriophages (Todd, 2000).

1.3.4. REPLICATION

As is true for most viruses, BFDV depends heavily on the host-cell DNA replication machinery for replication and is shown to replicate best in rapidly dividing tissues (Todd, 2000). According to Ritchie *et al.* (1991a), the gastrointestinal tract may be the site of replication and excretion of BFDV.

Rolling circle replication of DNA is characteristic of viruses possessing circular, ss-DNA genomes or ss-DNA intermediates in their replication cycles (Niagro, 1998).

The first step in replication of a circovirus ss-DNA genome is synthesis of the complementary strand to generate the first RF after which further DNA replication proceeds using the RCR mechanism. Bacteriophage ϕ X174 is the best known example of RCR: A virus-coded protein, A-protein, cleaves the virus strand DNA present in the

RF at a unique site, providing a 3'-OH terminus which acts as a primer and becomes extended by cellular DNA polymerase. The original virus strand is displaced as elongation of the 3'-OH terminus proceeds. After one round of replication the virus-coded protein (A-protein) cleaves the displaced virus strand from the newly synthesized strand and self-ligates to the displaced strand to form a circular ss-DNA molecule. A result of RCR is that many copies of the circular virus strand can be produced and can be used as template for complementary strand synthesis to generate more RF molecules or they can be encapsidated into virus particles (Todd *et al.*, 2001).

A common feature of ss-genomes is a potential stem-loop structure that contains a conserved nonanucleotide motif (TAGTATTAC) at its apex. The nonanucleotide motif is thought to be the initiation point of RCR (Ritchie *et al.*, 2003), as mutations of the first two nucleotides result in total loss of replicational function (Todd *et al.*, 2001). Adjacent to the potential stem-loop of BFDV is the occurrence of two repetitions of an eight base pair motif (GGGGCACC) (Niagro *et al.*, 1998), which based on similarities observed with the geminivirus and tomato golden mosaic virus may be the binding sites for the circovirus replication associated proteins (Mankertz *et al.*, 2000).

1.3.5. GENETIC DIVERSITY

Differences in clinical and pathological manifestation of PBFD in psittacine species have been thought to be due to host factors rather than antigenic or genetic variation of the virus (Ritchie *et al.*, 1990). According to Bassami *et al.* (2001), it is possible that adaptation of particular genotypes to certain species may have occurred or that regional differences in strains may have developed. These differences have significance in the understanding of the replication of BFDV and in the potential gene-coding assignments of the virus.

Comparative analysis of nucleotide sequences of the ORF1 (encoding the Rep protein) region of 10 BFDV isolates showed 88 – 89% identity between isolates, which according to Ypelaar and co-workers (1999) suggested the presence of only a single

genetic type of BFDV with the possibility of genomic variation outside of ORF1. Sequence analysis of BFDV performed by Bassami *et al.* (1998) (Australia) on an isolate designated BFDV-AUS showed sequence similarity (92%) to a genomic sequence derived from pooled BFDV (Niagro *et al.*, 1998) in the USA. The differences observed between these isolates were not major and it was suggested that there might be minimal sequence diversity between strains of BFDV world wide as was the case for APV (Johne and Müller, 1998).

In PCV (which is related to BFDV) two genotypes were reported (Meehan *et al.*, 1997; Hamel *et al.*, 1998). It was deemed a possibility that similar genotypic differences occurred in BFDV which might be detected if additional isolates of the virus were examined. This led to the study and comparison of eight BFDV strains from various regions throughout Australia from a variety of psittacine birds (Bassami *et al.*, 2001). It was found that all eight isolates investigated had the same basic structure, including the position of the ORFs, the location of the stem-loop structure, the nonanucleotide motif and the motifs within ORF1 involved in RCR and the P-loop motif. Genome size varied for the isolates and was due to a number of small deletions and insertions when compared to BFDV-AUS as described by Bassami *et al.*, 1998. The overall nucleotide identity of the isolates ranged from 84 – 97% and no evidence was found of distinctly different genotypes.

Due to the 73% variation of ORF2 (CP) and the different start codons for this ORF (ATG, CTG or TCT) as well as the differing positions of these start codons, it was suggested that intra-species antigenic variation might occur within the CPs (Bassami *et al.*, 2001) of BFDV. Raue and co-workers (2003) analyzed partial nucleotide sequences of the CP gene from 40 different DNA samples of BFDV and found that sequences obtained from an outbreak of Pbfd in lorries (*Trichoglossus sp.*) clustered in a separate branch of a phylogenetic tree and sequences from African Grey parrots with feather disorders grouped together, whereas those from the same species with immunosuppression clustered in other branches. Based on these results they suggested the existence of a particular BFDV genotype that preferentially infects the

cells of the feather follicles in African Grey parrots. Ritchie *et al.* (2003) suggested that all variants of BFDV might infect all psittacine species, but only certain genotypes are pathogenic in a certain species group.

In a study using Restriction Fragment Length Polymorphism (RFLP) analysis of ORF1 in South African BFDV isolates, the occurrence of different RFLPs were demonstrated, which could be an indication that there is more than one pathogenic strain of BFDV (Albertyn *et al.*, 2004).

Heath and co-workers (2004) obtained the nucleotide sequences of 10 BFDV isolates from diverse psittacine species at different geographical locations in South Africa and aligned them with previously published sequences. They found that Southern African isolates displayed the same basic genomic structures as was previously described for BFDV by Niagro *et al.* (1998), including the positions of the ORFs and the stem-loop structure located between the Rep and CP genes. In a study by de Kloet and de Kloet (2004), the complete size of the genomes of 15 different isolates of BFDV varied within narrow margins (1989 nts – 2019 nts) and there were differences in the size of sequence elements between segments encoding the Rep and CPs, which led to the conclusion that many different genetic strains of BFDV exist.

The level of genetic diversity among South African BFDV isolates are similar to those described in Australia and New Zealand. South African isolates have diverged from viruses found in other parts of the world and they have clustered into three unique genotypes. The existence of the sub-populations suggests that BFDV was introduced into Southern Africa on three separate occasions (Heath *et al.*, 2004).

With the movement of birds across geographical borders, the risk is increasing for spreading the disease to new areas and populations. There is also the added risk of the development of new unique viruses through recombination between the established virus populations and the newly introduced viruses. Recombination contributes

significantly to the genetic diversity of BFDV and is a common feature of the disease (Heath *et al.*, 2004).

1.3.6. PROTEINS AND ANTIGENS

BFDV consists of three structural proteins of 26.3 kDa, 23.7 kDa and 15.9 kDa as well as proteins which have a molecular weight of 60 kDa (Ritchie *et al.*, 1989; Ritchie *et al.*, 1990). Ritchie *et al.*, (1990) suggested that BFDV purified from numerous genera of diseased birds was similar, based on ultrastructural characteristics, protein composition and antigenic reactivity.

The theory that an antigenically related virus caused Pbfd in various psittacine genera was strengthened when virus purified from an umbrella cockatoo induced Pbfd in an umbrella cockatoo and an African Grey parrot. Further, a vaccine made from β -propiolactone-treated BFDV purified from a Moluccan cockatoo induced immunity in an umbrella cockatoo and an African Grey hen, which produced chicks that remained normal after viral challenge (Ritchie *et al.*, 1992a).

The CP of BFDV is a major constituent of viral particles and is a likely target of immune surveillance. The variability of specific sites within this protein could be the result of immune evasion (Heath *et al.*, 2004). The CP of BFDV has characteristics which exceeds its primary role in the encapsidation of viral particles. The CP is able to withstand high environmental temperatures of 80 - 85°C (Raidal and Cross, 1994) and is presumed to be responsible for cell surface receptor mediated attachment and entry into the susceptible cells. According to Heath and co-workers (2006), when the CP of BFDV is expressed in insect cells it is actively localized to the nucleus by one or more of three bipartite nuclear localization signals situated at the N-terminus of the protein. In addition to this, the authors also hypothesize that the CP directly interacts with Rep enabling co-translocation into the nucleus to establish active infection.

Ritchie *et al.*, 1990 found no strain variation in BFDV isolates from four different genera of psittacine birds with PBFD, using ultrastructural characteristics, protein composition and antigenic comparison. Similar sequence results by Niagro *et al.*, (1998) and Bassami *et al.*, (1998) support the hypothesis that there is only one strain of BFDV worldwide. The diversity within the genetics of the virus has not been related to antigenic differences. Determination of antigenic variation in BFDV is difficult due to the inability to cultivate the virus *in vitro*. However, Shearer *et al.*, 2008 conducted a study on BFDV in cockatiels and the authors suggest the presence of an antigenically distinct BFDV that is adapted to cockatiels.

1.4. CONCLUSIONS

PBFD is a severe dermatological condition in parrots which affects the survival of psittacine species worldwide. The causative agent of the disease is BFDV, a small circular ss-DNA virus belonging to the family *Circoviridae*. The virus immunocompromises the host by targeting the lymphoid tissue, thus making the birds susceptible to a variety of secondary bacterial and fungal infections. Normally these infections lead to the death of the birds.

PBFD is listed by the Australian Commonwealth Government as a key threatening process for the survival of five endangered psittacine species in Australia and in South Africa the survival of the indigenous, endangered Cape parrot is threatened. The need for effective control measures is apparent.

The inability to cultivate the virus *in vitro* in tissue/cell culture has hindered the development of a vaccine as well as studies into the genetics, antigenicity and pathogenicity of the virus. Thus far it has been assumed that there is only one strain of BFDV infecting psittacine birds. However, it has been found that the virus displays a high degree of genetic diversity within the CP gene. It is important to determine whether these genetic differences within the gene relates to antigenic differences in the virus.

With the loss of 10 – 20% of breeding stocks annually in South Africa alone, the development of a commercial vaccine is of the utmost importance. To achieve this goal successfully, it is necessary to determine whether there are different strains of BFDV infecting psittacine birds in order to achieve effective protection against all strains of the virus.

CHAPTER 2: INTRODUCTION TO THE PRESENT STUDY

Psittacine beak and feather disease (PBFD) is a common viral disease of wild psittacine birds in Australia, where one species is already endangered with extinction (Bassami *et al.*, 2001). PBFD is now found worldwide, including in South Africa where it threatens the survival of the indigenous endangered Cape parrot (*Poicephalus robustus*).

The most reliable test available for the diagnosis of PBFD is the polymerase chain reaction (PCR), but unfortunately this test is not quantitative and there is the occurrence of false negative results. There is no standardized serological test available with which to diagnose PBFD, this is due to the fact that *Beak and feather disease virus* (BFDV) can not be cultivated *in vitro*.

There is no commercially available vaccine which can aid in prevention of the disease and there is no cure for PBFD. The number of psittacine birds infected with BFDV will not decline unless the disease can be combated. The bird trade industry will continue to suffer monetary losses as well as the losses of valuable birds.

BFDV has been shown to group into different genotypes and many studies have been done which investigate the genetic variation of the virus. It has been established that BFDV is a genetically diverse virus, but no serotypes have been identified. No studies have been conducted to link the occurrence of the various genotypes to the existence of more than one strain of BFDV.

It is imperative to establish whether these genetic differences do cause antigenic variation in the virus, as vaccine development and the development of standardized

serological tests are based on the assumption that there is only one strain of BFDV that infects psittacine birds.

It therefore is the aim of this study to attempt to establish whether or not genetic variation within BFDV isolates led to antigenic variation by subjecting amino acid sequence data of the full length coat protein (CP) of BFDV to *in silico* analysis. It will also be attempted to express the full length CP genes in a bacterial expression system in order to analyse these genetic differences and its link to antigenicity *in vitro*.

CHAPTER 3:
**SEQUENCING AND *IN SILICO* ANTIGENIC PREDICTIONS OF THE COAT
PROTEIN GENES OF DIFFERENT *Beak and feather disease virus*
ISOLATES**

3.1. INTRODUCTION

Psittacine beak and feather disease (PBFD) is a fatal dermatological condition in parrots which is caused by *Beak and feather disease virus* (BFDV) (Shoemaker *et al.*, 2000) and it affects wild and captive psittacine birds worldwide.

The differences in clinical and pathological manifestation of PBFD was thought to be due to only host factors rather than genetic or antigenic variation of the virus (Ritchie *et al.*, 1990), but it is possible that the adaptation of particular genotypes to certain species may have occurred or that regional differences in strains have developed (Bassami *et al.*, 2001). With the movement of birds across geographical borders the risk is increasing for spreading the disease to new areas and populations. Also, there is the added risk of generating new and unique viruses through recombination as it is documented to be a key strategy for generating diversity in ribonucleic acid (RNA) and DNA viruses (Sambrook *et al.*, 2001) and it appears to contribute to the level of genetic diversity of BFDV (Ritchie *et al.*, 1989; Heath *et al.*, 2004).

Numerous studies have been done which investigate the genetic diversity of BFDV in Australia (Bassami *et al.*, 2001; Raue *et al.*, 2003), New Zealand (Ritchie *et al.*, 2003) and South Africa (Heath *et al.*, 2004, Kondiah *et al.*, 2004). These studies have shown genetic variation in both the Rep (ORF1) and coat protein (CP, ORF2) genes of BFDV. Phylogenetic analysis of the CP genes of BFDV isolates in South Africa revealed eight

BFDV lineages with Southern African isolates clustering into mainly three unique genotypes (Heath *et al.*, 2004).

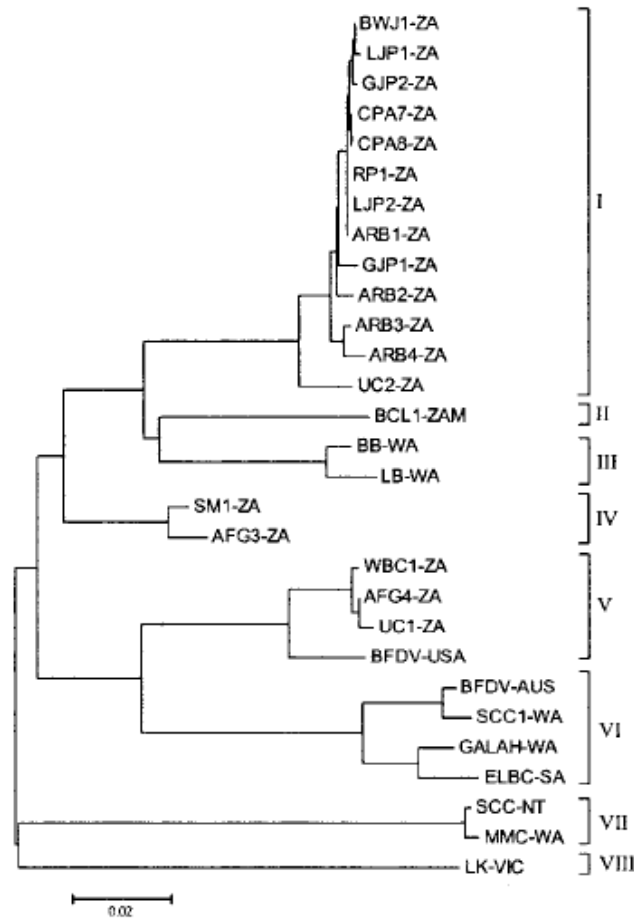


Figure 3.1: Maximum-likelihood tree indicating the presence of eight lineages of BFDV (Heath *et al.*, 2004).

Although it has been shown that there is genetic diversity within the CP genes of different BFDV isolates, little has been done to relate these genetic differences of the CP genes to antigenic variation. It is necessary to relate these two aspects, as vaccine development and the development of serological tests are based on the assumption that the CP contains the virus epitope and is the target for immunosurveillance. It is important to determine whether a vaccine produced from one genetic type will produce the correct antibodies in order to be effective against all variants of the virus. If it is shown that genetic variation does relate to antigenic variation, it is possible that a

vaccine based on one genetic type might not be effective in the fight against BFDV infection.

In the present study, various CP genes obtained from different birds and locations were amplified and sequenced. The sequence data was applied in an *in silico* study to tentatively predict if there is a possibility of antigenic variation amongst different isolates of BFDV.

3.2. MATERIALS AND METHODS

3.2.1. AMPLIFICATION OF THE COAT PROTEIN GENES

Various DNA samples were obtained from a variety of PBFV positive birds in the Free State (FS) and KwaZulu Natal (KZN), a full description of the samples can be seen in Table 3.1.

Table 3.1: Table indicating the DNA samples used in this study and their origin.

SAMPLE NAME	COMMON NAME	PSITTACINE SPECIES	SPECIES ORIGIN	SOURCE
DGR	African Grey parrot	<i>Psittacus erithacus</i>	Africa	Unknown
JKH	Jardine	<i>Poicephalus gularis massaicus</i>	Africa	KZN
LBR	Lovebird	<i>Agapornis roseicollis</i>	Africa	FS
PAR	Ring neck parakeet	<i>Psittacula krameri</i>	India	FS
RBKH	Red bellied parrot	<i>Poicephalus rufiventris</i>	Africa	KZN
KWS	Ring neck parakeet	<i>Psittacula krameri</i>	India	FS

Primers (Table 3.2) were designed and analysed using an algorithm from Integrated DNA Technologies (IDT) to amplify the entire CP gene (ORF2: nts 1234 – 1980 of the genome). Isolate AY450443 (GenBank) was used as template to design the primers. The restriction sites *NheI* and *XhoI* were incorporated at the 5' ends of the primers to facilitate ligation into the pET vector system from Novagen in future protein expression studies.

Table 3.2: Table indicating the primers designed for amplification of the CP gene, restriction sites are indicated in red boldface type.

PRIMER	SEQUENCE	SIZE (bp)	T _A (°C)	RESTRICTION ENZYME
PETCP – F	5' C CGCTAGC CCTGTGGGGCACCTCTAA 3'	24	69	<i>NheI</i>
PETCP – R	5' GTCTTTA CTCGAG TTAAGTACTGGGATTGTTGGG 3'	34	68	<i>XhoI</i>

Each polymerase chain reaction (PCR) reaction consisted of 5 µL of viral DNA as template, 1 µL of 10 mM dNTPs, 0.5 µL of each 100 mM primer, 5 µL of 10x concentration of ThermoPol Buffer (New England Biolabs®) and 3.75 U of Taq DNA Polymerase (New England Biolabs®). The reaction was made up to a final volume of 50 µL with sterile Milli-Q (MILLIPORE) water. Negative controls were performed which did not contain DNA template.

Reactions were thermocycled on a Mastercycler Personal (Eppendorf®). Initial denaturing was performed at 96°C for 5 minutes (min), after which denaturing was carried out at 96°C for 30 seconds (s), annealing was carried out at 63°C for 30 s and extension at 72°C for 90 s for 32 cycles. A final extension step was performed at 72°C for 10 min to allow complete elongation of product.

3.2.2. ANALYSIS OF PCR AMPLICONS

PCR amplicons were electrophoresed and visualised on 1% agarose gels containing GoldView (PEQLABS). Agarose gels were prepared and electrophoresed in TAE buffer (0.1 M Tris, 0.05 M EDTA [pH 8.0] and 0.1 mM glacial acetic acid) at 90 V for 35 min. The electrophoresed products were visualised with a ChemiDoc XRS (Bio-Rad Laboratories) under short wavelength ultra violet (UV) light.

3.2.3. SEQUENCING OF THE AMPLIFIED COAT PROTEIN GENES

Samples that displayed clear bands after PCR amplification and contained at least 100 ng DNA was selected for sequencing at Inqaba Biotec™.

Sequence data was assembled using *Vector NTI Advance™ 9.0* software from Informax™ which is a product of Invitrogen™ life science software. Analysed sequences were compared with known sequences in the GenBank Database using nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool, <http://ncbi.nlm.nih.gov>) to ensure that the sequences obtained showed similarity to known BFDV isolates. The nucleotide sequences were aligned using DNAssist 3.0 to compare identity with each other. Nucleotide sequences were translated using *Vector NTI Advance™ 9.0* software and translated sequences aligned with DNAssist 3.0.

3.2.4. PHYLOGENETIC ANALYSIS OF TRANSLATED SEQUENCES

The translated sequences were applied in a phylogenetic evaluation where MEGA3 software was used to construct a neighbour-joining phylogenetic tree using probability distance (p-distance). Isolates from this study, as well as isolates used by Heath *et al.*, 2004, were used in construction of this tree. The phylogenetic tree was rooted using the non-psittacine avian circoviruses; canary circovirus (GenBank accession number AJ301633), goose circovirus (GenBank accession number AJ304456) and columbid circovirus (GenBank accession number AJ298229).

3.2.5. SECONDARY STRUCTURE PREDICTIONS

β-bends may form part of the antigenic determinants of a protein (Hopp and Woods, 1981), thus by establishing where the bends occur one would have a better idea of where the antigenic sites of a protein would be. In order to obtain an idea of where possible antigenic sites of the CP could be; a prediction of the secondary structures of the CPs was undertaken. The crystal structure of the CP of BFDV has not been

established, thus before choosing a program to obtain the secondary structure of the proteins, the programs had to be analysed to ascertain which one would be best to predict secondary structure from amino acid sequence data.

This was done by analysing three proteins of which the crystal structure information is known and whose structures are in the Protein Data Bank (PDB). The proteins used for analysis was an oxidoreductase enzyme from *Candida albicans* (PDB 1AI9), bovine serine protease (PDB 1AB9) and aspartyl protease from human immunodeficiency virus 1 (PDB 1A8K). Secondary structure predictions were performed using SSpro, SOPMA, nnPREDICT and HNN from the ExPASy website (<http://au.expasy.org/tools#secondary>).

3.2.6. ANTIGENIC PREDICTIONS

The antigenicity of the isolates used in this study was predicted *in silico* with DNAssist 3.0. Antigenicity is predicted using an algorithm which utilizes solvent parameter values (s) for each amino acid that were assigned by Levitt in 1976 (as cited by Hopp and Woods, 1981). The solvent parameter value is the hydrophilicity value of the amino acid residue and is related to the energy required to transfer the residue from a hydrophobic to an aqueous environment (Patterton and Graves, 2000).

Hydrophilicity analysis of the protein is then carried out by the following method: Each amino acid in the protein sequence is assigned a hydrophilicity value; these values are then repetitively averaged down the length of the polypeptide chain, which then generates a series of local hydrophilicity values (Hopp and Woods, 1981). The antigenicity profile is then obtained in a graph of hydrophilicity versus the residue number of the amino acid sequence.

3.3. RESULTS AND DISCUSSION

3.3.1. IDENTIFICATION OF AMPLIFIED PRODUCTS

Primers were designed to amplify to the full length CP gene of BFDV, the template being isolate AY450443 from the GenBank database. The expected size of the PCR products amplified with the PETCP primers was approximately 770 base pairs (bp). Figure 3.2 indicates the amplified products obtained from the PETCP primer set.

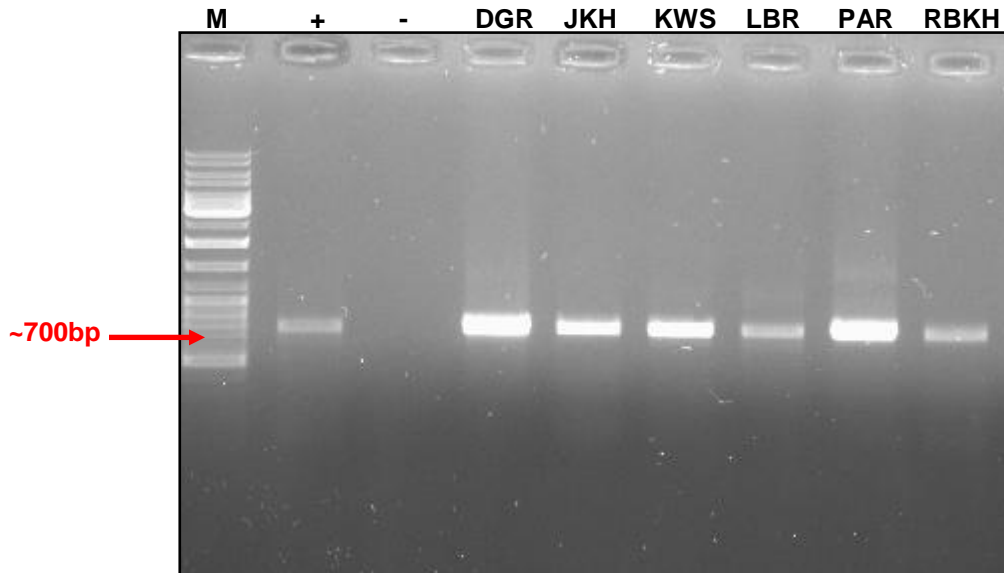


Figure 3.2: PCR amplified products using PETCP-F and PETCP-R primers to amplify the CP gene of BFDV, products are about 770 bp in size. Lane M represents the molecular marker (O'GeneRuler™ Ladder mix from Fermentas). Fragments were separated on a 1% w/v agarose gel with GoldView (PEQLABS) and visualised with UV illumination.

The samples depicted in Figure 3.2 were selected for sequencing at Inqaba Biotec™, where after the data obtained was applied in *in silico* analysis of the CP.

3.3.2. SEQUENCING OF AMPLIFIED PRODUCTS

Nucleotide-nucleotide BLAST for all the sequences showed that the isolates from this study had greater than 90% identity to other isolates of BFDV (Table 3.3).

Table 3.3: Table indicating nucleotide-nucleotide BLAST results for all the sequenced samples with isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCE	ISOLATE / SPECIES	ACCESSION NO	% IDENTITY
DGR	PK01 / <i>Psittacula kramerii</i>	AY521234	94%
	<i>Psephotus haemogaster</i>	AF311295	93%
JKH	ARB1-ZA	AY450452	100%
	RP1-ZA / <i>Poicephalus rueppellii</i>	AY450439	99%
	CPA7 / <i>Poicephalus robustus</i>	AY450438	99%
	CPA8-ZA / <i>Poicephalus robustus</i>	AY450437	99%
KWS	UC2-ZA	AY450450	99%
	HHP1-ZA	DQ397815	98%
	ARB1-ZA	AY450452	98%
	RP1-ZA / <i>Poicephalus rueppellii</i>	AY450439	98%
	CPA7 / <i>Poicephalus robustus</i>	AY450438	98%
	CPA8-ZA / <i>Poicephalus robustus</i>	AY450437	98%
LBR	HHP1-ZA	DQ397815	97%
	ARB1-ZA	AY450452	97%
	RP1-ZA / <i>Poicephalus rueppellii</i>	AY450439	97%
	CPA7 / <i>Poicephalus robustus</i>	AY450438	97%
	CPA8-ZA / <i>Poicephalus robustus</i>	AY450437	97%
	UC2-ZA	AY450450	97%
PAR	LJP2-ZA	AY450444	97%
	HHP1-ZA	DQ397815	97%
	ARB1-ZA	AY450452	97%
	RP1-ZA	AY450439	97%
	CPA7	AY450438	97%
	CPA8-ZA	AY450437	97%
RBKH	UC2-ZA	AY450450	97%
	ARB1-ZA	AY450452	100%
	RP1-ZA	AY450439	99%
	CPA7 / <i>Poicephalus robustus</i>	AY450438	99%

JKH	1	CGCTAGCCTGTGGGGCACCTCTAAC	TGCGCATGCGCTATATTT	CAGATTAGACGACGTAGATATG	65
RBKH	1	CGCTAGCCTGTGGGGCACCTCTAAC	TGCGCATGCGCTATATTT	CAGATTAGACGACGTAGATATG	65
KWS	1	CGCTAGCCTGTGGGGCACCTCTAAC	TGCGCATGCGCTATATTT	CAGATTAGACGACGTAGGTATG	65
LBR	1	CGCTAGCCTGTGGGGCACCTCTAAT	TGCGCATGCGCTATATTT	CCGATTAGACGACGTAGATATG	65
PAR	1	CGCTAGCCTGTGGGGCACCTCTAAC	TGCGCATGCGCTATATTT	CAGATTAGACGACGTAGATATG	65
DGR	1	CGCTAGCCTGTGGGGCACCTCTAAC	TGCGCATGCGCTATATTT	CAGATTAGACGACGTAGATATG	65
JKH	66	CCCGCCCATACTACCGCAGACGTCACA	ACAGGCGATACCGCCGAAGACGTAGATACT	TCCGCAGA	130
RBKH	66	CCCGCCCATACTACCGCAGACGTCACA	ACAGGCGATACCGCCGAAGACGTAGATACT	TCCGCAGA	130
KWS	66	CCCGCCCATACTACCGCAGACGTCACA	ACAGGCGATACCGCCGGAGACGTAGATACT	TCCGCAGA	130
LBR	66	CCCGCCCATACTGCCACCGCAGACGTCACAT	CAGGCGATACCGCCGGAGACGTAGATACT	TCCGCAGA	130
PAR	66	CCCGCCCATACTACCGCAGACGTCACA	ACAGGCGATACCGCCGGAGACGTAGATACT	TCCGCAGA	130
DGR	66	CCCGCCCATACTACCGCCGACGTCACAT	CAGGCGATACCGCCGGCAGGTAGATACT	TCCGCAAA	130
JKH	131	CGCCGTTTCTCAACCAATAGAATTT	TACACTATCAGATTCAAACGCCAATT	CAAATTCGAGATTTCG	195
RBKH	131	CGCCGTTTCTCAACCAATAGAATTT	TACACTATCAGATTCAAACGCCAATT	CAAATTCGAGATTTCG	195
KWS	131	CGCCGTTTCTCGACCAATAGAATTT	TACACTATCAGATTCAAACGCCAATT	CAAATTCGAAATTCA	195
LBR	131	CGCCGTTTCTCAACCAATAGAATTT	TACACTATGAGATTCAAACGCCAATT	CAAATTCGAAATTCA	195
PAR	131	CGCCGTTTCTCAACCAATAGAATTT	TACACTATGAGATTCAAACGCCAATT	CAAATTCGAAATTCT	195
DGR	131	CGCCGTTTCTCAACCAATAGGATTT	TACACTCTCAGATTAAAACGCCAATT	CAAATTTGAAATTCT	195
JKH	196	AAAGCAAACCACCAGTACTGGCCAAGTA	AATTTGGAATTTCTGACTACATAACCTTT	TGCATTGTCAG	260
RBKH	196	AAAGCAAACCACCAGTACTGGCCAAGTA	AATTTGGAATTTCTGACTACATAACCTTT	TGCATTGTCAG	260
KWS	196	AAAGCAAACCACCAGTACTGGCCAAGTA	AATTTGGAATTTCTGACTACATAACCTTT	TGCATTGTCAG	260
LBR	196	GAAGCAAACCACCAGTACTGGCCAAGTA	AATTTGGAATTTCTGACTACATAACCTTT	TGCATTGTCAG	260
PAR	196	GAAGCAAACCTACCCGTGCTGGACAAGTA	AATTTGGAATTTCTGACTACATAACCTTT	TGCATTGTCAG	260
DGR	196	GAAACAAACTACTCAAGCTGGACAAGT	GATTTGGAATTTCTGACTACATAACCTTTT	CATTGTCAG	260
JKH	261	ACTTCCTAGCAACAGCGCCAAACCCACATA	CATACTAAATTTCGAAGACTACCGGATTA	TAAATTAGCT	325
RBKH	261	ACTTCCTAGCAACAGCGCCAAACCCACATA	CATACTAAATTTCGAAGACTACCGGATTA	TAAATTAGCT	325
KWS	261	ACTTCCTAGCAACAGCGCCAAACCCACATA	CATACTAAATTTCGAAGACTACCGGATTA	TAAATTAGCT	325
LBR	261	ACTTCCTAGCAACAGCGCCAAACCCACATA	CATACTGAATTTCGAAGACTACCGGATTA	TAAATTAGCT	325
PAR	261	ACTTCCTAGCAACAGCGCCAAACCCACATA	CATACTGAATTTCGAAGACTACCGGATTA	TAAATTAGCT	325
DGR	261	ACTTCCTAGCAACAACCCGAACCCACATA	CATACTGAATTTCGAAGACTACCGTATTA	TAAATTAGCT	325
JKH	326	AAAATGGAATTGAGGCCACATGGGGCCACT	TACACAATTAGTGCAGACGGATT	CGGCCACACGGC	390
RBKH	326	AAAATGGAATTGAGGCCACATGGGGCCACT	TACACAATTAGTGCAGACGGATT	CGGCCACACGGC	390
KWS	326	AAAATGGAATTGAGGCCACATGGGGCCACT	TACACAATTAGTGCAGACGGATT	CGGCCACACGGC	390
LBR	326	AAAATGGAATTGAGGCCACATGGGGCCACT	TACACAATTAGTGCAGACGGATT	CGGCCACACGGC	390
PAR	326	AAAATGGAATTGAGGCCACATGGGGCCACT	TACACCATTAGTGCAGACGGATT	CGGCCACACGGC	390
DGR	326	AAAATGGAATTGAGGCCACATGGGGCCACT	TACACCATTAGTGCAGACGGATT	CGGCCACACGGC	390
JKH	391	CATAATCCAAGATTCCAGAATAACTAAATTT	TAAGACTACTACAGACCAA	ACTCAAGACCCAGTGG	455
RBKH	391	CATAATCCAAGATTCCAGAATAACTAAATTT	TAAGACTACTACAGACCAA	ACTCAAGACCCAGTGG	455
KWS	391	CATAATCCAAGATTCCAGAATAACTAAATTT	AAAACTACTTCAGACCAA	AAATCAAGACCCGGTGG	455
LBR	391	CATAATCCAAGATTCCAGAATAACTAAATTT	AAAACTACTTCGGACCAA	AAATCAAGACCCGGTGG	455
PAR	391	CATAATCCAAGATTCCAGAATAACTAAATTT	AAAACTACTTCGGACCAA	AAATCAAGACCCGGTGG	455
DGR	391	CGTAATCCAAGATTCCAGAATAACTAAATTT	AAAACTACTGCAGACCA	ATCAAGACCCAGTGG	455

Figure 3.3: Multiple sequence alignment of the amplified products from the CP gene (ORF2) of BFDV showing the areas with high percentage identity of nucleotide sequences. The forward arrow indicates the forward primer PETCP-F and the beginning of the amplified region, whereas the reverse arrow indicates the reverse primer PETCP-R and the end of the amplified region.

JKH	456	CTCCTTTTCGACGGTGCAAAAAAGTGGTATGTTGCCAGGGGATTCAAACGCCTCCTCAGACCGAAA	520
RBKH	456	CTCCTTTTCGACGGTGCAAAAAAGTGGTATGTTGCCAGGGGATTCAAACGCCTCCTCAGACCGAAA	520
KWS	456	CTCCTTTTCGACGGTGCAAAAAAGTGGTATGTTGCCAGGGGATTCAAACGCCTCCTCAGACCGAAA	520
LBR	456	CTCCTTTTCGACGGTGCAAAAAAGTGGTATGTTGCCAGGGGATTCAAACGCCTCCTCAGACCGAAA	520
PAR	456	CTCCTTTTCGACGGTGCAAAAAAGTGGTATATTGCCAGGGGATTCAAACGCCTCCTCAGACCGAAA	520
DGR	456	CACCTTTTCGACGGTGCCAAGAAATGGTACCTTAGCAGGGGATTCAAACGCCTGCTCAGACCGAAA	520
JKH	521	CCTCAAATAACTATAGATGACCTGACCACTGCGAACCAGTCTGCGGCATTGTGGCTAAACAGCGC	585
RBKH	521	CCTCAAATAACTATAGATGACCTGACCACTGCGAACCAGTCTGCGGCATTGTGGCTAAACAGCGC	585
KWS	521	CCTCAAATAACTATAGATGACCTGACCACTGCGAACCAGTCTGCGGCATTGTGGCTAAACAGCGC	585
LBR	521	CCTCAAATAACTATAGACGACCTGACCACTGCGAACCAGTCTGCGGCATTGTGGCTAAACAGCGC	585
PAR	521	CCTCAAATAACTATAGATGACCTGACCACTGCGAACCAGTCTGCGGCATTGTGGCTAAACAGCGC	585
DGR	521	CCACAAATAACAATAGATGACCTGACCACTGCAAACCAGTCTGCGGCATTGTGGCTAAACAGCGC	585
JKH	586	CAGAACCGGCTGGATCCCCTACTACAAGGAGGACCCAATGCGGCAGGAGCGAACGTCAAGCACTACG	650
RBKH	586	CAGAACCGGCTGGATCCCCTACTACAAGGAGGACCCAATGCGGCAGGAGCGAACGTCAAGCACTACG	650
KWS	586	CAGGACCGGCTGGATCCCCTACTACAAGGAGGACCCAATGCGGCAGGAGCGAACGTCAAGCACTACG	650
LBR	586	CAGAACCGGCTGGATCCCCTACTACAAGGAGGACCCAATGCGGCAGGAGCGAACGTCAAGCACTACG	650
PAR	586	CAGAACCGGCTGGATCCCCTACTACAAGGAGGACCCAATGCGGCAGGAGCGAACGTCAAGCACTACG	650
DGR	586	CAGAACCGGCTGGATCCCCTACTACAAGGAGGACCCAACGCGGCAGGAGCGAACGTCAAGCACTACG	650
JKH	651	GCCTTGCATTTCAGCTTCCCTCAGCCGGAAGTGACAATCACTTATGTATGCGGAGCTCACTCTGTAT	715
RBKH	651	GCCTTGCATTTCAGCTTCCCTCAGCCGGAAGTGACAATCACTTATGTATGCGGAGCTCACTCTGTAT	715
KWS	651	GCCTTGCATTTCAGCTTCCCTCAGCCAGAAGTGAAAATCACTTATGTATGCGGAGCTCACTCTGTAT	715
LBR	651	GCCTTGCATTTCAGCTTCCCTCAGCCTGAAGTGAAAATCACTTATGTATGCGGAGCTCACTCTGTAT	715
PAR	651	GCCTTGCATTTCAGCTTCCCTCAGCCAGAAGTGAACATCTCTTATGTATGCGGAGCTCACTCTGTAT	715
DGR	651	GCCTTGCCTTCAGCTTCCCTCAGCCAGAAGTAAAGATCTCTTATGTATGTGAGCTGACTTTGTAT	715
JKH	716	GTTCAATTCCGTCAGTTTGCCCCCAACAATCCCAGTACTTAACTCGAGTAAAGAC	770
RBKH	716	GTTCAATTCCGTCAGTTTGCCCCCAACAATCCCAGTACTTAACTCGAGTAAAGAC	770
KWS	716	GTTCAATTCCGTCAGTTTGCCCCCAACAATCCCAGTACTTAACTCGAGTAAAGAC	770
LBR	716	GTTCAATTCCGTCAGTTTGCCACCAACAATCCCAGTACTTAACTCGAGTAAAGAC	770
PAR	716	GTTCAATTCCGTCAGTTTGCCCCCAACAATCCCAGTACTTAACTCGAGTAAAGAC	770
DGR	716	GTTCAATTCCGTCAAATTTGCCCCCAACAATCCCAGTACTTAACTCGAGTAAAGAC	770



Figure 3.3 continued: Multiple sequence alignment of the amplified products from the CP gene (ORF2) of BFDV showing the areas with high percentage identity of nucleotide sequences. The forward arrow indicates the forward primer PETCP-F and the beginning of the amplified region, whereas the reverse arrow indicates the reverse primer PETCP-R and the end of the amplified region.

The nucleotide sequences were aligned with DNAssist 3.0. The alignment of the sequences showed many areas of identity, with base differences only in a few areas (Figure 3.3). These sequences were translated to observe the amino acid composition and to subject the CP gene sequences to further analysis in order to hypothesize whether or not differences in genetic composition are significant in the antigenicity of the protein.



Figure 3.4: Protein sequence alignment of sequenced products show a high percentage of identity (>93%). The black arrows indicate the putative bipartite nuclear localization signal (NLS) which correlates with findings described by Heath *et al.*, 2006.

The translation of the sequences (Figure 3.4) revealed that isolates JKH and RBKH were 100% identical, this was expected as these two isolates, although from different psittacine species were isolated from the same farm in KZN. The putative bipartite NLS as described by Heath and co-workers in 2006 can be observed in all the isolates, the NLS aids in actively localizing the CP of BFDV into the nucleus of cells. Although the NLS at the N-terminus of the CP of BFDV isolates is a conserved region, there are a few amino acid substitutions that can be observed when the sequences of these isolates are aligned, these substitutions may or may not contribute to the antigenic character of the proteins.

The sequence data obtained from the isolates used in this study was submitted to GenBank, the accession numbers are listed below in Table 3.4.

Table 3.4: Table indicating the isolates from this study and their corresponding GenBank accession numbers.

ACCESSION NO	ISOLATE	COMMON NAME
EU624326	DGR	African Grey parrot
EU624327	JKH	Jardine
EU624328	LBR	Lovebird
EU624324	PAR	Ring neck parakeet
EU624325	RBKH	Red bellied parrot
EU624329	KWS	Ring neck parakeet

After aligning the sequences and establishing that there were amino acid differences between the isolates, a neighbour-joining phylogenetic tree was constructed (Figure 3.5) in MEGA3 using probability distance (p-distance). The phylogenetic tree was constructed to determine where the isolates used in this study grouped within the phylogenetic tree constructed by Heath and co-workers (2004), this particular tree described the eight genotypes (lineages) into which different BFDV isolates grouped.

3.3.3. PHYLOGENETIC ANALYSIS

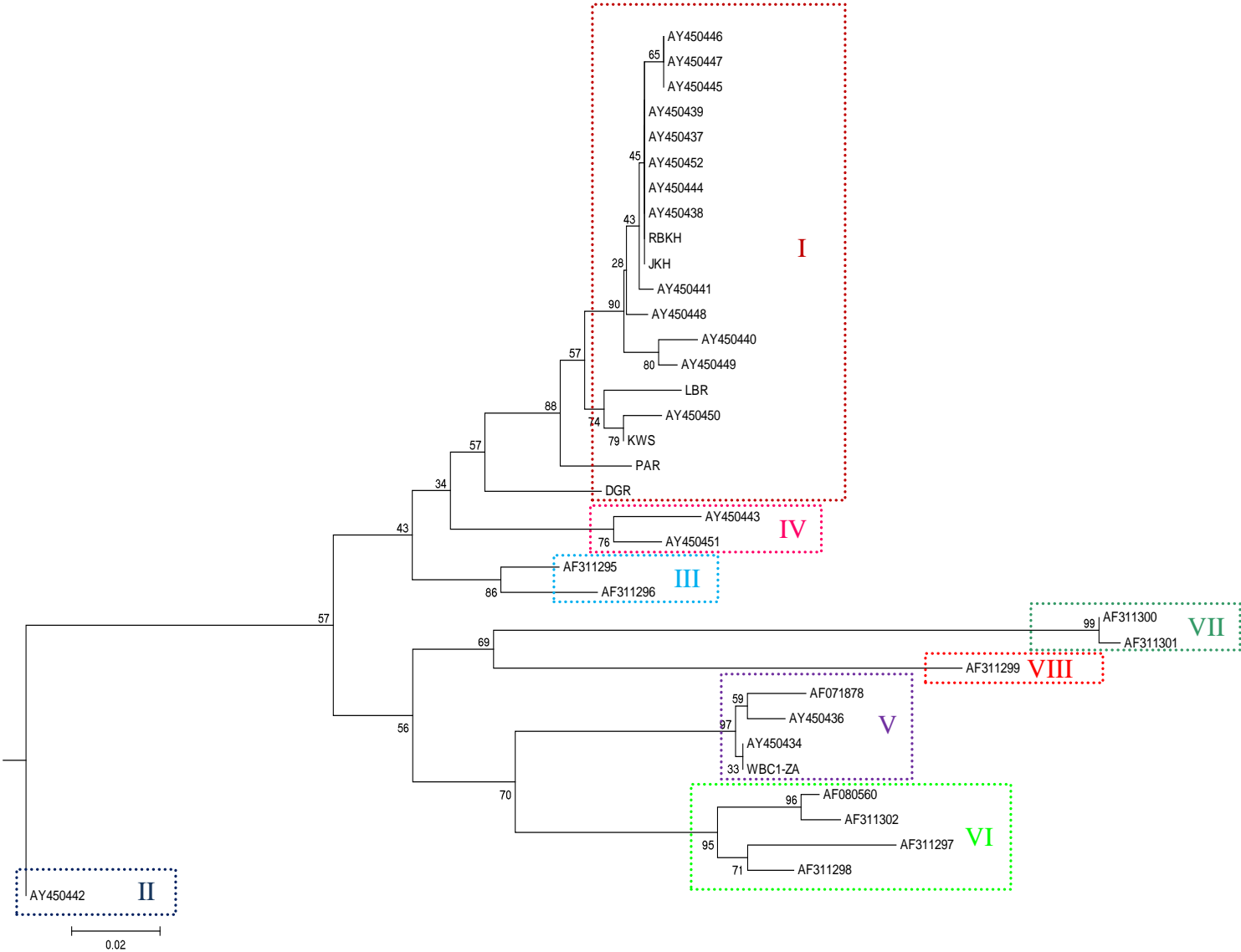


Figure 3.5: A neighbour-joining phylogenetic tree of the isolates used in this study as well as the isolates described by Heath *et al.*, 2004. The different lineages are depicted by the different colour boxes, the phylogenetic analysis reveals that all the isolates used in this study fall within the first lineage. The tree was rooted using the 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 containing the BFDV isolates is shown here.

The isolates from this study fall within the first group of the described lineages, which is not surprising as lineage I, II and IV are described by Heath and co-workers (2004) to be the three unique genotypes into which Southern African isolates group. Lineage V also contains Southern African isolates which are closely related to isolates from North America, it is unclear whether this is a newly introduced genotype in South Africa or whether it is representative of a fourth genotype that evolved in South Africa and spread to other parts of the world (Heath *et al.*, 2004).

3.3.4. SECONDARY STRUCTURE ANALYSIS

The analysis for secondary structure prediction of the programs SSpro, SOPMA, nnPREDICT and HNN showed that predicting secondary structure from sequence data alone is not reliable.

The secondary structure data obtained from the abovementioned programs was compared with the crystal structures of the three proteins analysed. It was found that the programs did predict some of the secondary structures correctly, but unfortunately extra structural information was predicted where it did not occur in the crystal structures and some structures were completely left out (data not shown). Not one prediction program could be isolated with accuracy to predict the secondary structure of the BFDV CP with.

Even though predictions have become more accurate over time, from the results found by comparing the findings of the programs with the actual crystal structures of the three proteins (PDB 1A19, PDB 1AB9 and PDB 1A8K) it can be seen that structural prediction programs still have a long way to go before predicting secondary structure with accuracy, as there are a lot of environmental, chemical, physical and physiological interactions and factors which have to be brought into consideration to mimic the environment which causes a protein to fold and interact in a certain way.

Predictions of secondary or tertiary structures would be more accurate if the CP amino acid sequence of BFDV showed a higher degree of homology to proteins whose structures have been solved and is in the PDB. Homology modelling is a computational method for the modelling of a structure of a protein based on its sequence similarity to one or more proteins of known structure (Petsko and Ringe, 2004).

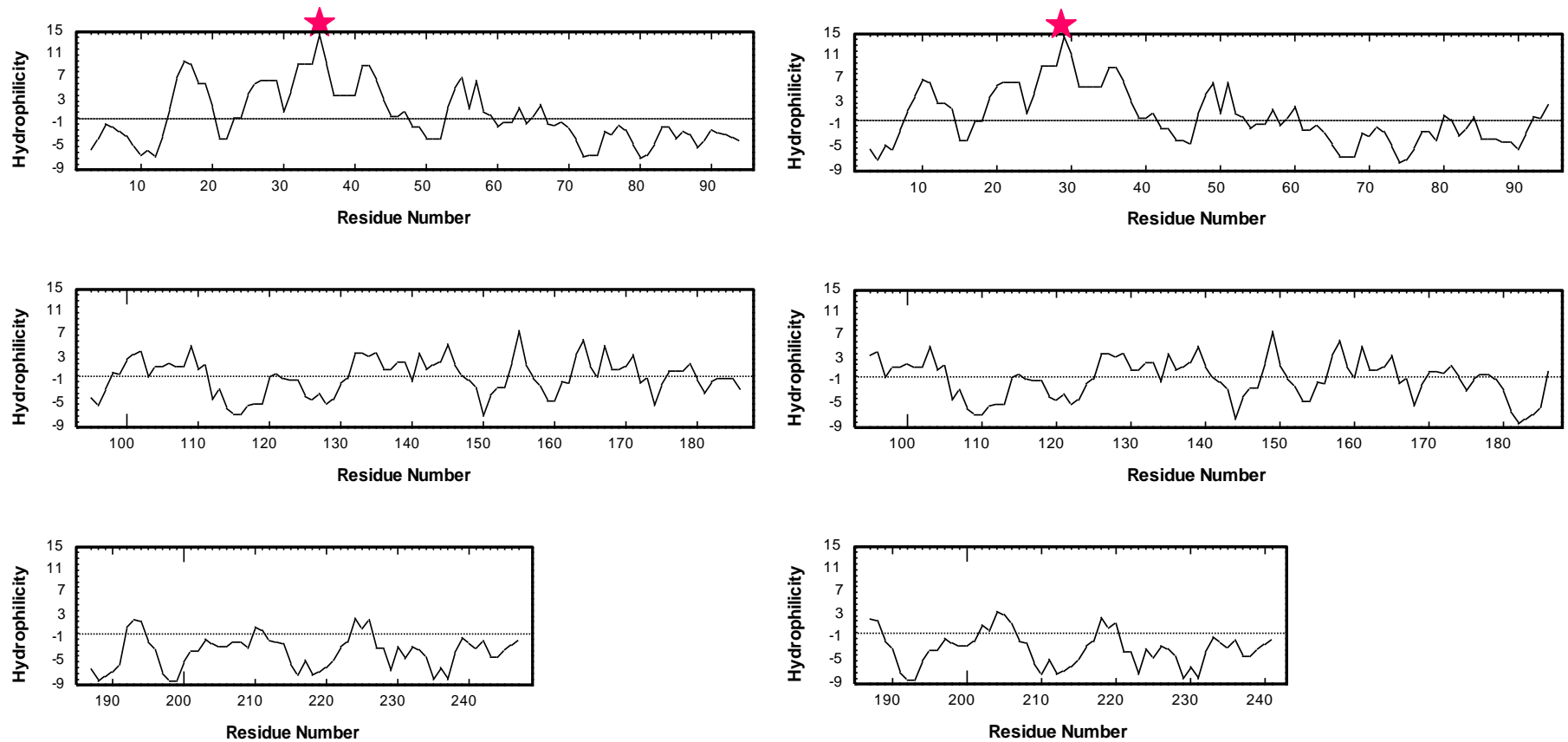
3.3.5. ANTIGENIC PREDICTIONS

Studies have demonstrated that antigenic determinants are surface features of a protein and that they are mostly found on regions of a molecule with a high degree of exposure to solvent, thus projecting into the medium (Hopp and Woods, 1981). Hopp and Woods (1981) stated that charged hydrophilic amino acid side chains are common features of antigenic determinants and suggested that the point of highest local average hydrophilicity is located in, or immediately adjacent to an antigenic determinant.

Even though the secondary structures of the BFDV CPs was not predicted, *in silico* analysis of the CPs was continued by predicting possible antigenic profiles with the use of the amino acid sequence data of the proteins. To predict antigenicity each amino acid was assigned a hydrophilicity value (Table 3.5). Antigenic profiles of hydrophilicity versus residue number were predicted for the CPs with DNAssist 3.0 using the default program settings (Figure 3.6).

Table 3.5: Table indicating the solvent parameter values (s, hydrophilicity values) assigned to each amino acid by Levitt (as cited by Hopp and Woods, 1981).

AMINO ACID	s, kcal/mol (Hydrophilicity value)	AMINO ACID	s, kcal/mol (Hydrophilicity value)
Arginine	3.0	Alanine	-0.0
Aspartic acid	2.5	Histidine	-0.5
Glutamic acid	2.5	Cysteine	-1.0
Lysine	3.0	Methionine	-1.3
Serine	0.3	Valine	-1.5
Asparagine	0.2	Isoleucine	-1.8
Glutamine	0.2	Leucine	-1.8
Glycine	0.0	Tyrosine	-2.3
Proline	-1.4	Phenylalanine	-2.5
Threonine	-0.4	Tryptophan	-3.4

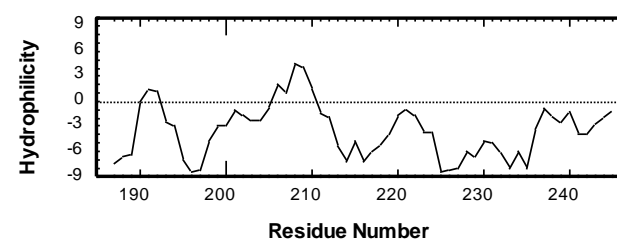
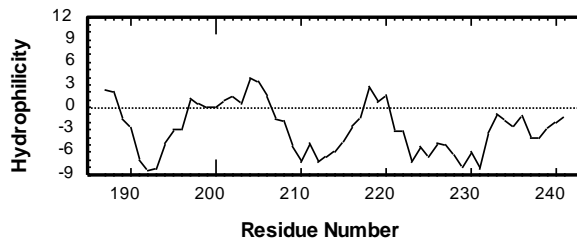
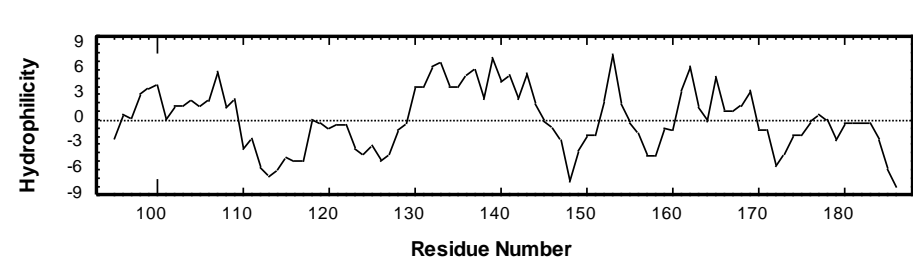
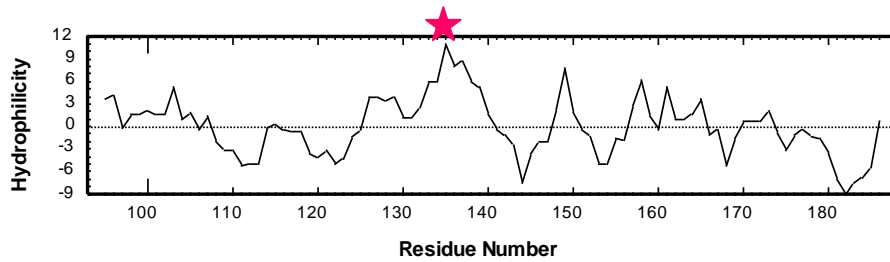
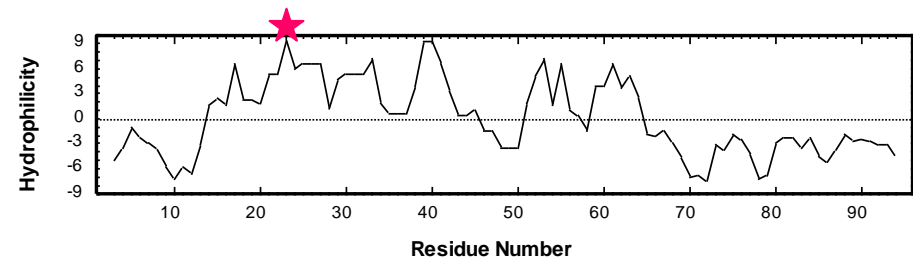
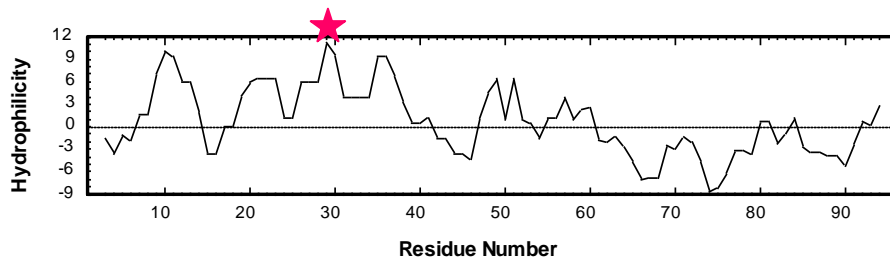


a. Profile representative of lineages I, IV and V

b. Profile representative of lineages III, VI and VII

Figure 3.6: Antigenicity profiles (hydrophilicity versus residue number) for (a) isolates from lineages I, IV and V, (b) isolates from lineages III, VI and VII, (c) isolate AY450442 and (d) AF311299 (Heath *et al.*, 2004). The averaged antigenicity values are plotted versus sequence position of the amino acids. Data points are plotted at the centre of the averaging group from which they were derived.

★ Indicate the highest point of hydrophilicity on the profiles, which is likely to be an antigenic determinant or immediately adjacent to an antigenic determinant on the proteins.



c. BCL1-ZAM (AY450442)

d. LK-VIC (AF311299)

Figure 3.6 continued: Antigenicity profiles (hydrophilicity versus residue number) for (a) isolates from lineages I, IV and V, (b) isolates from lineages III, VI and VII, (c) isolate AY450442 and (d) AF311299 (Heath *et al.*, 2004). The averaged antigenicity values are plotted versus sequence position of the amino acids. Data points are plotted at the centre of the averaging group from which they were derived.

★ Indicate the highest point of hydrophilicity on the profiles, which is likely to be an antigenic determinant or immediately adjacent to an antigenic determinant on the proteins.

Antigenic profiles based on the hydrophilicity values of the amino acids were obtained for the isolates from this study as well as for one isolate from each of the other lineages (genotypes).

Two unique isolates that each have their own genotype and fall into their own lineages are isolates BCL1-ZAM (lineage II) and LK-VIC (lineage VIII). The isolate from lineage II is the endangered black-cheeked lovebird (*Agapornis nigrigenis*) from Zambia (Figure 3.6c, BCL1-ZAM / AY450442) and the isolate from lineage VIII is a rainbow lorikeet (*Trichoglossu haematodus*) from Australia (Figure 3.6d, LK-VIC / AF311299).

According to findings by Hopp and Woods (1981), one antigenic determinant is consistently located at the point of maximum hydrophilicity. All the isolates from lineage I (including the isolates from this study) displayed the same antigenic profile (Figure 3.6a) with the highest hydrophilicity value approximately at position 35 of the amino acid sequences. The isolates from lineage IV and V displayed the same antigenic profile as those from lineage I (data not shown), with a few subtle variations in the profile; however, the highest hydrophilicity peak remained at ~35. It is possible that this high peak at ~35 is an antigenic determinant of the CP of BFDV; this high peak is a result of the group of Arg residues at the N-terminus of the CP, which have the highest solvent parameter (hydrophilicity) values.

The Australian BFDV CP isolates from lineages III, VI and VII all displayed similar antigenic profiles (Figure 3.6b) to the profiles obtained for the South African isolates (Figure 3.6a). The highest peak for the Australian isolates was also obtained at ~35.

Isolate BCL1-ZAM displayed an antigenic profile (Figure 3.6c) that was different from the profiles obtained in Figure 3.6a and b. For BCL1-ZAM the profile showed two high peaks with the same hydrophilicity values at both ~35 and ~135. It is possible that either one or both of these peaks represent an antigenic determinant for this isolate.

Isolate LK-VIC showed high hydrophilicity values at position ~25 (Figure 3.6d) which were higher than the value at ~35. It is possible that this peak can constitute an antigenic site on this protein that is different from the South African and Australian isolates.

Another major difference between the hydrophilicity plots were obtained approximately between residues 132 – 143, where isolates from the South African and Australian lineages (Figure 3.6a and b) had general hydrophilicity values ranging between -1 and 3, in LK-VIC (Figure 3.6d) the hydrophilicity values increased and ranged between 2 and 7. With isolate BCL1-ZAM the value increased significantly and averaged between 0 and 11, the highest peak at ~135 (Figure 3.6c).

When comparing the antigenic profiles of hydrophilicity versus residue number for all the isolates from the different lineages, it appears that both the South African and Australian (Figure 3.6a and b) isolates have the same possible antigenic determinant sites at the same site on the CPs. The profiles obtained for these isolates are very much alike and suggests that even though these isolates fall into different genotypes on the phylogenetic tree, they may not necessarily differ antigenically.

Heath and co-workers (2004) proposed a theory that all BFDV isolates evolved from a common ancestor that may have originated in Africa and then spread to Australasia. The authors also suggested that the lineage belonging exclusively to lorikeets (lineage VIII) could represent the “true Australian variant” of BFDV. They found no recombinant regions within the genome of the Zambian isolate (lineage II) and suggested that separate and unique Southern African genotypes may exist. This *in silico* study of antigenicity of BFDV isolates supports the theory of Heath and co-workers (2004), in that both the Australian and South African isolates displayed the same antigenic profile (Figure 3.6a and b). The lineage unique to lorikeets produced an antigenic profile (Figure 3.6d) that was relatively similar to that of the South African and Australian isolates, but had differences where the antigenic sites were predicted for this isolate. The “uniqueness” of the Zambian isolate is displayed in the antigenic profile obtained for

it, as it's profile was unique to this isolate with major hydrophilicity values in more than one location, which could indicate an alternative antigenic site on the CP of this isolate. The antigenic profiles of isolates BCL1-ZAM and LK-VIC (Figure 3.6c and d, respectively) showed higher hydrophilicity values at different sites on the proteins, indicating the possibility of the presence of two antigenically distinct isolates.

From the data at hand, it can be hypothesized that genetic differences of the CP genes of BFDV does lead to antigenic variation of the proteins; although a specific genetic pattern contributing to antigenicity could not be established. However, this being an *in silico* application does not make it a fully reliable test of antigenicity as there is no secondary structures or crystal structure available for the CP of BFDV with which to compare the data with. Also, the antigenicity predictions performed in this study only accurately predict one antigenic determinant. Hopp and Woods (1981) found that one antigenic determinant was consistently located at the point of highest hydrophilicity, but not all antigenic determinants are associated with the high points of hydrophilicity and not all high points are associated with antigenic determinants. Thus, it is possible that the major predicted antigenic determinants are not antigenic determinants at all.

It is necessary to perform studies *in vitro* in order to determine whether there is more than one antigenic strain of BFDV. The ability to predict antigenic determinants from amino acid sequence data alone is potentially very useful, even though only a single determinant can be predicted with relative confidence. Once an antigenic determinant/region have been predicted, it is possible to verify its existence by targeting this region for synthesis and testing its activity in an appropriate immune assay. This approach is particularly useful for a virus such as BFDV which can not be cultivated *in vitro* and of which there is a limited amount of DNA available with which to perform investigative studies. According to Hopp and Woods (1981) antigenic prediction of pathogenic organisms can be useful in the production of synthetic vaccines against these strains.

3.4. CONCLUSIONS

BFDV has been shown to group into eight lineages based on the unique genotypes of the viruses. Even though the existence of these lineages has been shown, no studies have been done to relate the genetic differences of the viruses to possible antigenic variation of BFDV. Thus far it has been assumed that there is only one antigenic strain of BFDV worldwide.

The CP is a major constituent of infectious viral particles and is likely to be a target of immune surveillance (Heath *et al.*, 2004). Different serotypes of BFDV have not yet been identified, but it is a possibility that there are antigenically distinct groups of BFDV as was found by Shearer and co-workers (2008), who identified an antigenically distinct BFDV that was adapted to cockatiels.

In this study it was undertaken to sequence the CP gene of various isolates of BFDV that were of South African origin. The amino acid sequences were subjected to *in silico* analysis in order to predict whether there is the possibility that BFDV strains may differ antigenically. It was found that South African and Australian isolates presented with antigenic profiles that were the same. The lorikeet isolate and the black-cheeked lovebird isolates both presented with antigenic profiles that were unique to them and was not observed in any other isolates analysed. Based on the *in silico* analysis, it can be concluded that there is more than one strain of BFDV that infects psittacine species. However, it is important to note that *in silico* predictions are exactly that, the results found in this study have to be performed *in vitro* in order to confirm or deny the presence of antigenic variation of the virus.

It is important to establish whether there are antigenically distinct strains of BFDV as birds are moved across the borders frequently and there is the risk of generating unique viruses through recombination. Knowing whether there are different serotypes of BFDV is a very important aspect of vaccine development as well as the development of reliable diagnostic tests.

CHAPTER 4:
EXPRESSION OF GENETICALLY DIFFERENT COAT PROTEIN GENES OF
Beak and feather disease virus

4.1. INTRODUCTION

Attempts at cultivating *Beak and feather disease virus* (BFDV) *in vitro* in tissue/cell cultures and embryonated eggs have failed (Johne *et al.*, 2004). Lack of a culture system for the cultivation of BFDV has hampered investigation into vaccine development, establishment of serological diagnostic tests as well as studies into the antigenicity of the virus.

Open reading frame (ORF) 2 of BFDV encodes the coat protein (CP), which is a 26.3 kDA protein believed to be the epitopic protein able of inducing an immune response in diseased birds (Kondiah, 2008). Bassami and co-workers (2001) found that the CP genes under their investigation showed 73% variation. The high degree of genetic diversity within the CP gene has not been related to antigenic variation of the virus yet.

Recombinant expression of the antigenic protein of BFDV in numerous systems is now an attractive option with which to produce antigen, which will enable investigation into the antigenicity of the virus, as well as development of vaccines and diagnostic tests. A truncated form of the CP was successfully expressed by Johnes and co-workers (2004) in *Escherichia coli* with the pQE-60 vector. Heath *et al.*, 2006 successfully expressed the whole CP gene as well as a variety of truncated versions of this protein in a Bac-to-Bac baculovirus expression system in SF-21 insect cells.

E. coli based expression systems are popular for the production of heterologous protein expression, due to their ability to grow rapidly at high density on inexpensive substrates, as well as their well-characterized genetics and the availability of a large number of cloning vectors and mutant host strains (Terpe, 2006). *E. coli* is the most versatile host for the production of heterologous proteins although it has drawbacks which include the inability to perform many post-translational modifications found in eukaryotic proteins, the lack of a secretion mechanism which enables release of the protein into the culture medium and it has limited ability to facilitate disulfide bond formation (Jana and Deb, 2005).

The Novagen pET system is one of the first choices for protein expression in *E. coli*, the reason being that the target genes are cloned under control of the T7 promoter which is not recognised by the *E. coli* RNA polymerase. Virtually no expression will occur until a source of T7 RNA polymerase is provided, thus the genes cloned into the pET vectors are essentially “off” in non-expression hosts and cannot cause plasmid instability. Once established, plasmids are transferred into expression hosts containing the chromosomal copy of the T7 RNA polymerase gene and expression of genes are induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), such systems have been found to produce a high protein expression level and allow for the strong control of gene expression (Studier and Moffatt, 1986).

Expression hosts contain a prophage (λ DE3) encoding the enzyme under control of the promoter. This system leads to the synthesis of large amounts of mRNA and in most cases the accumulation of the desired protein at very high concentrations (40 – 50% of total cell protein), but it does have drawbacks, for example: a high level of mRNA can cause destruction of the ribosome and leaky expression of T7 RNA polymerase may result in plasmid or expression instability (Baneyx, 1999).

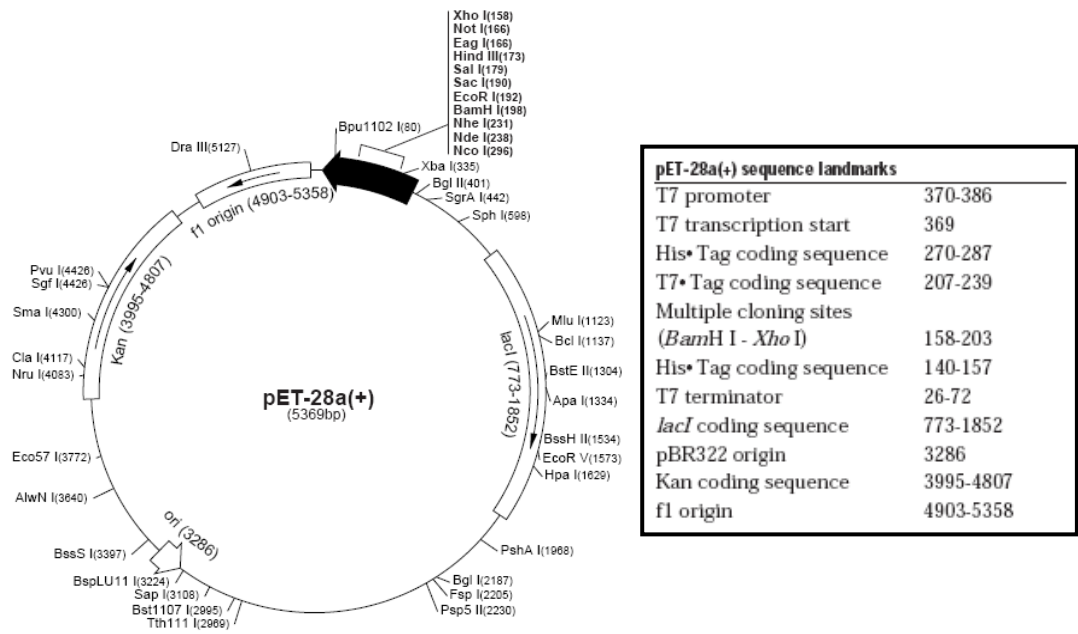


Figure 4.1: Schematic map showing the elements of the pET-28 vector (Novagen).

For the purpose of this study the pET-28b(+) vector (see Figure 4.1) was used to express the full length CP gene of six isolates of BFDV. The six isolates varied genetically and it was attempted to express the six proteins corresponding to these genes in order to investigate antigenicity *in vitro* with the use of an enzyme linked immunosorbent assay.

4.2. MATERIALS AND METHODS

4.2.1. PCR AMPLIFICATION OF THE COAT PROTEIN GENES

The CP genes of the six isolates discussed in Chapter 3 were PCR amplified with the PETCP primers (Table 3.2) which incorporated *NheI* and *XhoI* restriction sites on the 5' and 3' sides of the amplicons, respectively. These restriction sites were incorporated to facilitate ligation into the pET-28b(+) vector system from Novagen. The PETCP-R primer included a stop codon (TAA) that would prevent addition of the C-terminal six-histidine (6x His) tag.

Each PCR reaction consisted of 5 µL of viral DNA as template, 1 µL of 10 mM dNTPs, 0.5 µL of each 100 mM primer, 5 µL of 10x concentration of ThermoPol Buffer (New England Biolabs®) and 3.75 U of Taq DNA Polymerase (New England Biolabs®). The reaction was made up to a final volume of 50 µL with sterile Milli-Q water. Negative controls were performed in which the DNA template was excluded.

Reactions were thermocycled on a Mastercycler Personal (Eppendorf®). Initial denaturing was performed once at 96°C for 5 minutes min, after which denaturing was carried out at 96°C for 30 s, annealing was carried out at 63°C for 30 s and extension at 72°C for 90 s for 32 cycles. A final extension step was carried out at 72°C for 10 min to allow complete elongation of product.

4.2.2. ANALYSIS OF PCR AMPLICONS OR RESTRICTION ENZYME PRODUCTS

Amplicons were electrophoresed and visualised on 1% (w/v) agarose gels containing GoldView (PEQLABS). The agarose gels were prepared and electrophoresed in TAE buffer (0.1 M Tris, 0.05 M EDTA [pH 8.0] and 0.1 mM glacial acetic acid) at 90 V for

35 min. Visualisations were performed with a ChemiDoc XRS (Bio-Rad Laboratories) under short wavelength ultra violet (UV) light.

4.2.3. PURIFICATION OF DNA FROM AGAROSE GELS

For cloning purposes the amplified DNA was purified from the agarose gel with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

The amplified DNA was viewed and excised from the agarose gel with the use of a DarkReader™ transilluminator (Fermentas). The weight of the agarose gel band was determined and 10 µL of Capture buffer type 2 was added for every 10 mg of gel. The tubes were incubated at 60°C and mixed by inversion every 3 min until the agarose was completely dissolved. A volume of 600 µL of the Capture buffer type 2 sample mixture was transferred onto an assembled GFX MicroSpin column and Collection tube. The samples were incubated at room temperature (rt) for 1 min, followed by centrifugation at 16 000 x *g* for 30 s (Eppendorf 5415D). The flow through from the column was discarded and 500 µL of Wash buffer type 1 was added to the GFX MicroSpin column containing the bound DNA. The assembled column and collection tubes were centrifuged at 16 000 x *g* for 30 s. The GFX MicroSpin column was transferred to a sterile DNase-free 1.5 mL microcentrifuge tube, 30 µL of Elution buffer type 4 was added to the centre of the membrane and incubated at rt for 1 min. The assembled column and sample collection tube was centrifuged at 16 000 x *g* for 1 min to elute the purified DNA.

4.2.4. CLONING OF THE SIX AMPLIFIED ISOLATES INTO THE pGEM™TEasy VECTOR SYSTEM I.

4.2.4.1. TRANSFORMATION INTO TOP 10 *E. coli* COMPETENT CELLS

Gel purified DNA was ligated into the pGEM™TEasy vector (PROMEGA) in a 10 µL reaction. The reactions consisted of 5 µL purified DNA (3:1 ration of insert DNA to vector DNA), 1 µL of 50 ng/µL vector, 2 µL 2x ligation buffer and 5 U T4 DNA ligase (Fermentas). The reactions were incubated for 2 hours at rt before transformation into TOP 10 *E. coli* competent cells.

The TOP 10 *E. coli* cells were made competent with an adjusted version of the rubidium chloride (RbCl₂) method as described by Hanahan (1983) and used in 50 µL aliquots.

For each ligation reaction, 50 µL of competent cells were thawed on ice after which 10 µL of ligated plasmid was added to it. A negative control was included which consisted of competent cells with no plasmid. The mixture was placed on ice for 30 min, followed by 40 s at 42°C and placed on ice again for 2 min. A volume of 800 µL of pre-warmed SOC medium was added to the samples and shaken at 37°C for 1 hour. Samples were centrifuged for 30 s at full speed to pellet the cells and approximately 750 µL of the supernatant was aspirated. The cell pellet was resuspended in the remaining 100 µL of supernatant and plated out onto pre-warmed Luria Bertani (LB) plates that were supplemented with ampicillin (30 mg.mL⁻¹), IPTG (10 mg.mL⁻¹) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside [40 mg.mL⁻¹]). The plates were incubated for 16 hours overnight (O/N) at 37°C after which they were stored at 4°C.

4.2.4.2. CONFIRMATION OF INSERT DNA IN pGEM™TEasy

Positive transformants were selected from the plates, inoculated into 5 mL LB media containing 50 µL of 10 mg.mL⁻¹ ampicillin stock solution and incubated O/N for 18 hours at 37°C on a shaker.

Small-scale plasmid isolations were performed on the samples with the Zyppy™ Plasmid Miniprep Kit (ZYMO RESEARCH).

A volume of 600 µL of the O/N bacterial cultures was added into 1.5 mL microcentrifuge tubes, a 100 µL of 7x Lysis Buffer was added to the tubes and mixed by inversion 4 - 6 times, until the solution turned clear blue (indicating complete lysis). After lysis 350 µL of cold Neutralization Buffer was added and mixed thoroughly (complete neutralization was indicated by the solution turning yellow and the formation of a yellowish precipitate). The neutralized samples were centrifuged (Eppendorf 5415D) at full speed for 4 min. The supernatant was transferred to the Zymo-Spin™ II column, whilst avoiding disturbing the cell pellet. The columns were placed into collection tubes and centrifuged at full speed for 15 s, the flow through was discarded, 200 µL of Endo-Wash Buffer was added to the columns and centrifuged again at full speed for 15 s. Thereafter, 400 µL of Zyppy™ Wash Buffer² was added to the columns and the samples were centrifuged for 30 s at full speed. The columns were transferred to clean 1.5 mL microcentrifuge tubes and 30 µL of Zyppy™ Elution Buffer³ was added directly to the column matrix and incubated at rt for 1 min. The samples were centrifuged for 15 s to elute the plasmid DNA which was stored at -20°C until required.

Screening for positive recombinant plasmids was performed with restriction digests utilizing the restriction endonucleases *NheI* and *XhoI* (Fermentas). A double digest was performed on each sample; the reactions consisted of 5 µL plasmid DNA, 5 U *NheI*, 20 U *XhoI*, 1 µL 1x Buffer Tango™ (Fermentas) and 1.5 µL sterile Milli-Q water in a total reaction volume of 10 µL. The reactions were incubated in a 37°C waterbath for 2 hours and resolved on a 1% agarose gel containing GoldView.

DNA inserts were excised from the pGEM™TEasy vector by performing a double digest as described above on the positive clones. The digested samples were resolved on a 1% agarose gel stained with GoldView and the correct bands were purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), as described in section 4.2.3. The eluted DNA was stored at -20°C until required.

4.2.5. CLONING OF THE SIX ISOLATES INTO THE pET-28b(+) VECTOR

4.2.5.1. PREPARATION OF pET-28b(+) FOR LIGATION WITH INSERT DNA

Preparation of the 5' and 3' ends of the pET-28b(+) vector entailed restriction digestion of the vector with *NheI* and *XhoI* to linearise the plasmid. This also removed most of the multiple cloning site, but the ATG start codon at the *NcoI* restriction site and the N-terminal 6x His tag remained intact. A double digest was performed which consisted of 10 µL pET-28b(+) vector, 5 U *NheI*, 20 U *XhoI*, 3 µL 1x Buffer Tango™ and 12 µL sterile Milli-Q water in a reaction volume of 30 µL. The linearised vector was purified from a 1% agarose gel containing GoldView with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), as described in section 4.2.3.

After digestion and purification from the gel, the vector was dephosphorilated. The dephosphorilation reaction consisted of 50 µL pET-28b(+) vector, 6 µL 10x reaction buffer, 3 µL sterile Milli-Q water and 1 U of calf intestinal alkaline phosphatase (CIAP) in a total volume of 60 µL. The reaction was incubated at 37°C for 30 min after which the vector DNA was purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the purification from an enzymatic reaction protocol.

A volume of 500 µL of Capture buffer type 2 was added to the 60 µL dephosphorilation reaction and mixed thoroughly. The Capture buffer type 2 sample mix was centrifuged (Eppendorf 5415D) briefly to collect the liquid at the bottom of the tube. The entire Capture buffer type 2 sample mix was loaded onto an assembled GFX MicroSpin column and collection tube and centrifuged for 30 s at full speed. The flow through was discarded and 500 µL of Wash buffer type 1 was added to the GFX MicroSpin column after which the assembled column and collection tube was centrifuged for 30 s at full speed. The flow through was discarded and the GFX MicroSpin column was transferred to a clean 1.5 mL microcentrifuge tube and 30 µL of Elution buffer type 4 was added to the centre of the membrane and incubated at rt for 1 min. The assembled

column and microcentrifuge tube was centrifuged at full speed for 1 min to collect the purified DNA, which was stored at -20°C.

4.2.5.2. TRANSFORMATION INTO TOP 10 *E. coli* COMPETENT CELLS

Purified DNA from the six BFDV isolates were ligated into the prepared pET-28b(+) vector. Ligation reactions consisted of 5 µL DNA, 1 µL vector, 2 µL ligation buffer and 5 U T4 DNA ligase in a total volume of 10 µL. The ligation reactions were incubated at rt for 2 hours.

For each ligation reaction, 50 µL of TOP 10 *E. coli* competent cells were thawed on ice after which 10 µL of ligated plasmid was added to it. A negative control was included which consisted of competent cells with no plasmid. The mixture was placed on ice for 30 min, followed by 40 s at 42°C and placed on ice again for 2 min. A volume of 800 µL of pre-warmed SOC medium was added to the samples and shaken at 37°C for 1 hour. Samples were centrifuged (Eppendorf 5415D) for 30 s at full speed to pellet the cells and approximately 750 µL of the supernatant was aspirated. The cell pellet was resuspended in the remaining 100 µL of supernatant and plated onto pre-warmed LB plates that were supplemented with 30 mg.mL⁻¹ kanamycin. The plates were incubated for 16 hours O/N at 37°C after which they were stored at 4°C.

4.2.5.3. CONFIRMATION OF INSERT DNA IN pET-28b(+)

Colonies from each transformation was selected and inoculated into 5 mL LB media containing 50 µL of 10 mg.mL⁻¹ kanamycin stock solution. These samples were incubated O/N for 18 hours at 37°C on a shaker.

Small-scale plasmid isolations were performed on the samples with the Zyppy™ Plasmid Miniprep Kit (ZYMO RESEARCH) as described in 4.2.4.2. The eluted plasmid DNA was stored at -20°C until required.

To confirm the presence of the six isolates into the vector, restriction digests were performed with *NheI* and *XhoI* on each sample as described in 4.2.4.2. Restriction fragments were resolved on a 1% agarose gel stained with GoldView.

4.2.5.4. SEQUENCING OF RECOMBINANT DNA IN pET-28b(+)

The recombinant plasmid DNA was sequenced using the T7 promotor and T7 terminator (Fermentas) primers to ascertain that the DNA was inserted into the vector in the right orientation.

Sequencing reactions were prepared with the BigDye terminator v.3.1 kit. Plasmid DNA was amplified in a sequencing PCR using the T7 promotor and terminator primers. The reactions consisted of 0.25 μL Premix, 1 μL ($3.2 \text{ pmol. } \mu\text{L}^{-1}$) of either the T7 promotor or terminator primer, 2 μL dilution buffer, 100 – 500 ng of the plasmid DNA and sterile Milli-Q water up to a volume of 10 μL .

The reactions were thermocycled on the Mastercycler Personal (Eppendorf[®]) at 96°C for 1 minute followed by 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. A final cooling step of 4 °C was also included.

Post-reaction cleanup was performed with EDTA/Ethanol precipitation. The sequencing reaction volume was adjusted to 20 μL with sterile Milli-Q water and transferred to a 1.5 mL microcentrifuge tube containing 5 μL 125 mM EDTA and 60 μL absolute ethanol. The reactions were mixed by vortexing for 5 s and then precipitated at rt for 15 min. The tubes were centrifuged at 4°C for 10 min at 20 000 x *g* (Eppendorf[®] Centrifuge 5417 R). The supernatant was completely aspirated, making sure that the cell pellet was not disturbed. The samples were dried in a Speedvac Concentrator (SAVANT).

Sequencing was performed on an ABI Prism[®] Sequencer from Applied Biosystems.

4.2.5.5. SEQUENCE ANALYSIS

Sequence data obtained was assembled using *Vector NTI Advance*[™] 9.0 software from Informax[™] (Invitrogen[™] life science software). With the use of DNAssist 3.0 the sequences were aligned with the sequences obtained in Chapter 3. The alignments were performed in order to assure that no deletions or insertions had occurred and that the DNA cloned into the pET-28b(+) vector was in the correct orientation and in frame for subsequent protein expression.

4.2.6. EXPRESSION OF RECOMBINANT COAT PROTEIN IN BL21(DE3) *E. coli* COMPETENT CELLS

The BL21(DE3) *E. coli* strain (Novagen) has the following genotype: F- *ompT hsdS_B* (*r_B-m_B-*) *gal dcm* (DE3). The DE3 designation indicates that the strain contains the λDE3 lysogen that carries the gene for T7 RNA polymerase under control of the *lacUV5* promoter. IPTG is required to induce expression of the T7 RNA polymerase. The strain is an *E. coli* B/r strain that does not contain *lon* protease, the strain is also deficient of the outer membrane protease, OmpT. The lack of these two key proteases reduces degradation of heterologous proteins expressed (Novagen).

4.2.6.1. TRANSFORMATION INTO THE BL21(DE3) *E. coli* EXPRESSION HOST

After confirmation of the presence of insert DNA in the pET-28b(+) vector, the purified plasmid DNA was transformed into the expression host, BL21(DE3) *E. coli* competent cells. The transformation was performed as described in section 4.2.4.1 for five of the six isolates.

4.2.6.2. RECOMBINANT EXPRESSION OF COAT PROTEIN GENES

4.2.6.2.1. PREPARATION OF PRE-INOCULUM

The cloned CP genes of five of the BFDV isolates (DGR, KWS, LBR, PAR and RBKH) were used for protein expression. A white colony was picked up from every one of the transformed plates for all five of the samples. These colonies were used to inoculate 5 mL LB media supplemented with 50 μL of 10 $\text{mg}\cdot\text{mL}^{-1}$ kanamycin for each sample. The cultures were incubated at 37°C with shaking until $\text{OD}_{600} = 0.6 - 1.0$, after which they were stored O/N at 4°C. The following morning the cells were collected by centrifugation (Eppendorf 5415D) at full speed for 30 s, the supernatant was discarded and the cell pellet resuspended in 2 mL LB supplemented with 50 μL of 10 $\text{mg}\cdot\text{mL}^{-1}$ kanamycin.

4.2.6.2.2. EXPRESSION OF THE COAT PROTEIN GENES

The resuspended pre-inoculum was used to inoculate 50 mL LB media supplemented with 30 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin and incubated at 37°C with shaking until $\text{OD}_{600} = 0.6 - 1.0$. A 1 mL sample of cells was removed from every flask, centrifuged (Eppendorf 5415D) at maximum speed for 30 s and the supernatant discarded. The cell pellets were frozen at -20°C, this formed the pre-induction sample.

Two different concentrations of IPTG was used to induce expression of the proteins. The cultures were induced with either 0.4 mM or 1 mM final concentration IPTG and expression was carried out at 19°C O/N, 30°C for 8 hours and 37°C for 5 hours with aeration. Thereafter, 1 mL samples were harvested from the flasks during expression and centrifuged at maximum speed to pellet the cells. The supernatant was discarded and the cell pellets frozen at - 20°C for analysis.

The samples taken during expression were analysed by 10% sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) to determine where expression of the CP

occurred. Alternatively, the SuperSignal[®] West HisProbe[™] Kit (PIERCE) was used to determine whether the proteins were expressed.

4.2.6.3. ANALYSIS OF EXPRESSED PROTEINS WITH POLYACRYLAMIDE GEL ELECTROPHORESIS

The bacterial pellets collected during expression were thawed and resuspended in 300 μL of B-PER[®] Bacterial Protein Extraction Reagent (PIERCE) with the addition of protease inhibitor cocktail (PIERCE) by vigorously vortexing for 1 min. The tubes were shaken for 30 min at rt. The samples were centrifuged for 5 min at 15 000 $\times g$ to separate soluble proteins from insoluble proteins. The supernatant was transferred to a clean tube and this constituted the soluble fraction. The cell pellet was resuspended in 300 μL of B-PER[®] Reagent, which constituted the insoluble fraction.

Inclusion body purification was also performed where 6 μL lysozyme (10 $\text{mg}\cdot\text{ml}^{-1}$ in B-PER[®]) was added to the insoluble fraction and vortexed for 1 min. B-PER[®] was diluted 1:10 with sterile Milli-Q water and 1 mL was added to the suspension after which the mixture was vortexed for 1 min. The samples were centrifuged (Eppendorf[®] Centrifuge 5417R) at full speed for 10 min at 4°C, the pellet resuspended in 1 mL 1:10 diluted B-PER[®] and vortexed for 1 min. This procedure was repeated three times. After the last centrifugation step the inclusion body pellet was resuspended in 300 μL sterile Milli-Q water.

A 15 μL sample of the soluble, insoluble and inclusion body fractions were used to load the SDS-PAGE gels after the addition of an equal volume (15 μL) of Laemmli buffer containing β -mercaptoethanol (19:1). The samples were boiled for 10 min and loaded onto a 10% SDS-PAGE gel together with 5 μL of PageRuler[™] Prestained Protein Ladder or Spectra[™] Broad Range Protein Ladder (Fermentas). The gels were cast and run in a Mini-PROTEAN 3 Cell System (BIORAD) and run at 70 V for 30 min, after which it was adjusted to 100 V for 90 min. Gels were either stained for visualization or blotted O/N for use in Western blotting to detect the N-terminal 6x His tag.

Table 4.1: Composition of the 10% resolving and 4.5% stacking SDS-PAGE gels.

COMPONENT	RESOLVING (mL)	STACKING (mL)
Distilled water	5	3.187
Sample buffer (Resolving: 1.125 M Tris, pH 8.8; 0.3% SDS) (Stacking: 0.375 M Tris, pH 6.8; 0.3% SDS)	2.5	1.25
Acrylamide/Bisacrylamide	2.5	0.563
TEMED	10 μ L	5 μ L
Ammonium persulphate (APS, 10%)	100 μ L	50 μ L

Gels were stained and destained using the modified Fairbanks Coomassie Blue Protein Staining and Destaining Method (Fairbanks *et al.*, 1971) or they were stained O/N with PageBlue™ Protein Staining Solution (Fermentas).

After electrophoresis, gels were placed in a microwaveable container and approximately 100 mL of Fairbanks A staining solution (0.05% Coomassie Blue R-250, 25% isopropanol, 10% acetic acid) was added. The gels with the solution were heated in a conventional microwave oven on full power until boiling point was reached after approximately 2 min. The gel and solution was cooled to rt with gentle shaking for 5 min. The Fairbanks A solution was discarded and the gel rinsed with distilled water at rt for a few seconds. After this step, bands containing 100 ng or more were observed. Approximately 100 mL of Fairbanks B solution (0.005% Coomassie Blue R-250, 10% isopropanol, 10% acetic acid) was added and the process described above was repeated. After this step, protein bands containing 50 ng or more were visible. Approximately 100 mL of Fairbanks C solution (0.002% Coomassie Blue R-250, 10% acetic acid) was added to the gels and the process was repeated again, protein bands containing 25 ng or more was visible after this step. Lastly, approximately 100 mL of Fairbanks D solution (destaining solution [10% acetic acid]) was added to the gels and the process described above was repeated. After this process protein bands containing 5 ng became visible. A clear background was achieved by leaving the gels in the destaining solution for 15 min or more with gentle shaking.

4.2.6.4. WESTERN BLOTTING

Western blotting was performed in a Mini Trans-Blot Cell (BIORAD) after briefly washing the gel in transfer buffer (25 mM Tris [pH 8.3], 192 mM glycine, 20% methanol and 0.025 – 0.1% SDS). Protein was transferred O/N at 4°C to a HyBond ECL Nitrocellulose Membrane (GE Healthcare) at 30 V. Detection of protein bands was performed using the SuperSignal[®] West HisProbe[™] Kit (PIERCE).

4.2.6.5. PROTEIN DETECTION WITH THE SuperSignal[®] West HisProbe[™] KIT

The SuperSignal[®] West HisProbe[™] Kit combines the HisProbe[™]-HRP Technology and the SuperSignal[®] West Pico Chemiluminescent Substrate for the detection of proteins expressed in frame with a 6x His tag, independent of their location and adjacent sequences.

The nitrocellulose membrane was briefly rinsed in TBST (Tris Buffered Saline Tween[®]-20) and then blocked with 10 mL of BSA/TBST for 1 hour at rt with shaking. The membrane was washed twice with 15 mL of TBST for 10 min each on a shaker, after which it was incubated with 10 mL of HisProbe[™]-HRP Working Solution for 1 hour at rt with shaking. After incubation, the membrane was washed four times while on a shaker with 15 mL of TBST for 10 min each. Afterwards, the blot was incubated with 10 mL of SuperSignal[®] West Pico Substrate Working Solution for 5 min. The blot was placed in a membrane sheet protector, which was placed in a film cassette, exposed for 60 s and developed.

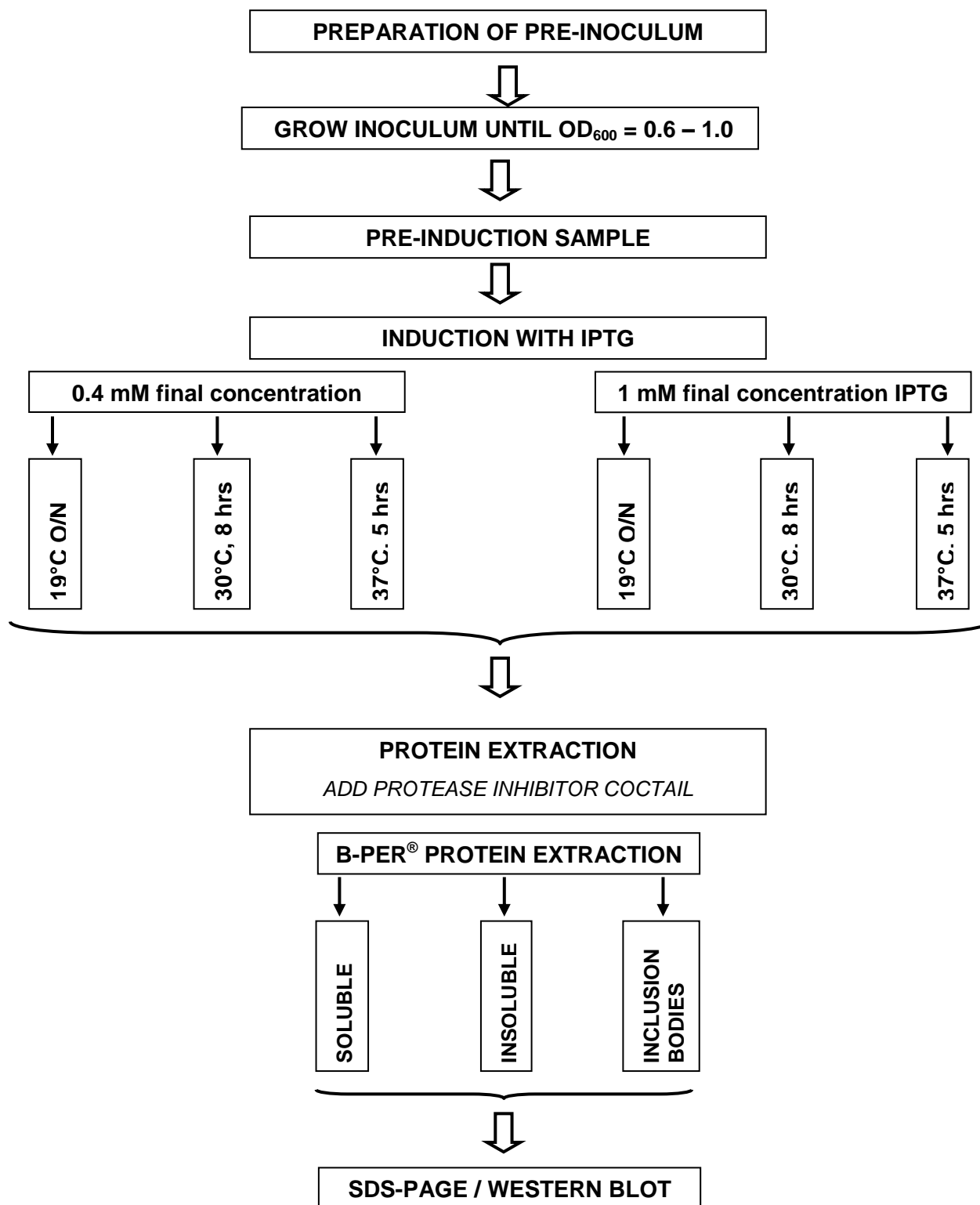


Figure 4.2: Experimental outlay of protein expression.

4.3. RESULTS AND DISCUSSION

4.3.1. IDENTIFICATION OF AMPLIFIED PRODUCTS

The six DNA isolates were amplified with the PETCP primer set which incorporated the *NheI* and *XhoI* restriction sites, the expected bands of the amplified products was approximately 770 bp in size. Figure 4.3 shows amplified DNA for all six of the isolates.

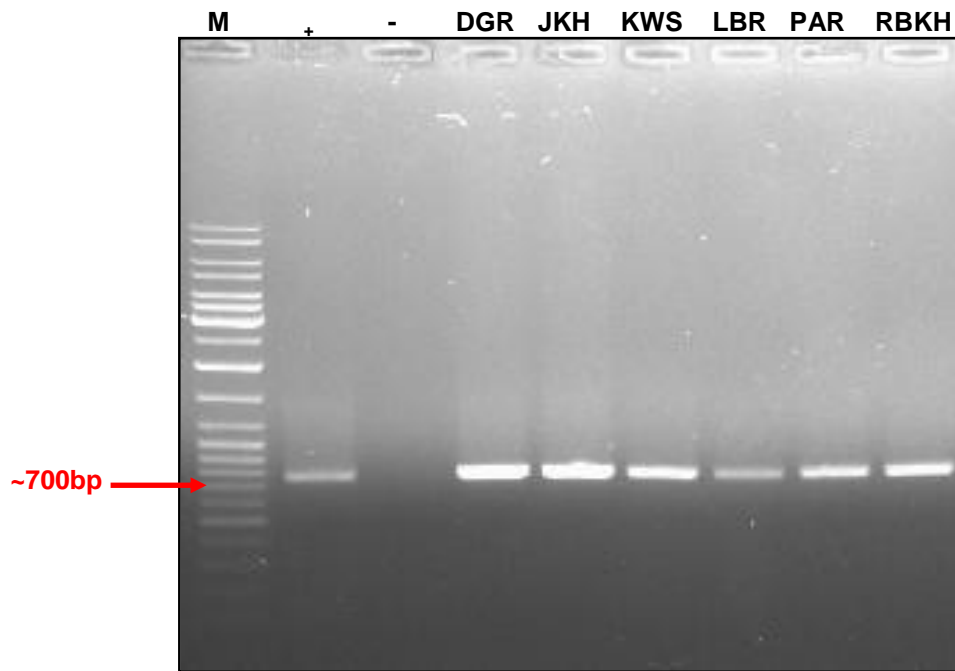


Figure 4.3: A 1% w/v agarose gel visualised under UV illumination showing the amplified CP genes of the six isolates. Lane M represents the O'GeneRuler™ DNA Ladder mix (Fermentas) molecular marker. Both positive and negative controls were included.

4.3.2. RESTRICTION PROFILES OF CLONED PRODUCTS

The gel-purified DNA was ligated into pGEM™TEasy and successfully transformed into TOP 10 *E. coli* competent cells. Small scale plasmid isolations were performed on selected colonies and the plasmid DNA was cleaved in a double digest utilizing the

restriction endonucleases *NheI* and *XhoI*. Positive clones yielded a restriction profile with bands at approximately 3000 bp and 770 bp, which comprises the vector backbone and the insert DNA respectively (Figure 4.4).

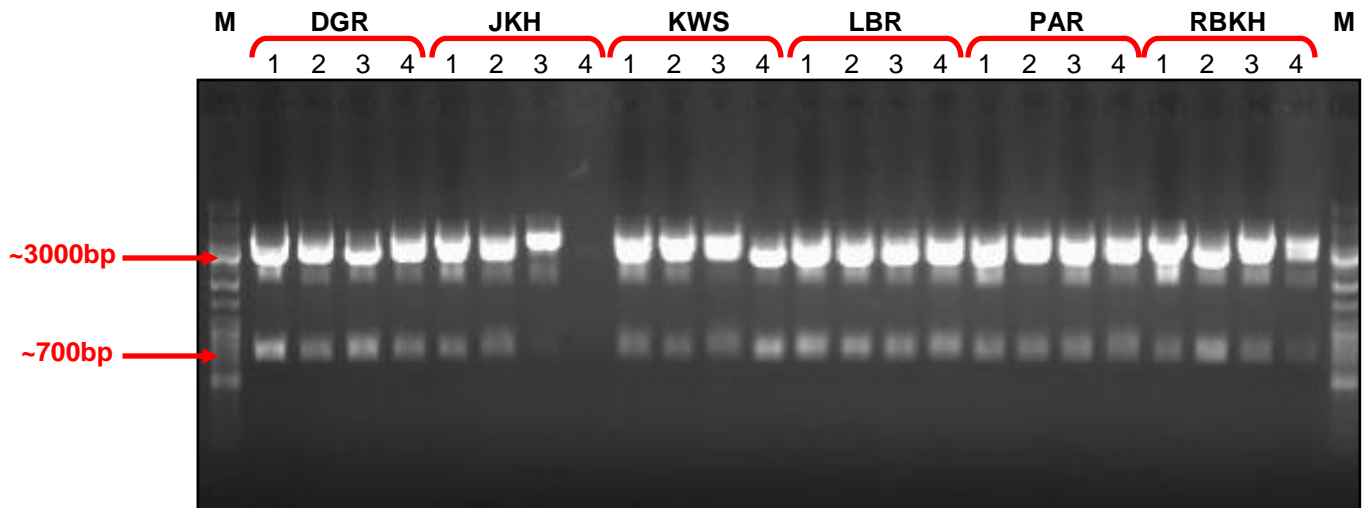


Figure 4.4: A 1% w/v agarose gel visualised under UV illumination showing the restriction profile of plasmid DNA digested with *NheI* and *XhoI*. At ~3000 bp is the pGEM™TEasy vector backbone and at ~770bp is the insert DNA. Only one of the isolated colonies did not show the presence of vector or insert DNA (lane 4, sample JKH). Lanes M represents the O'GeneRuler™ DNA Ladder Mix (Fermentas) molecular weight marker.

The DNA was cleaved from pGEM™TEasy with *NheI* and *XhoI* and ligated into the pET-28b(+) vector from Novagen which was used to transform TOP 10 *E. coli* competent cells. The N-terminal 6x His tag would be incorporated during translation; the reverse primer PETCP-R contained a stop codon, which prevented the 6x His tag from being added at the C-terminal.

Small scale plasmid isolations were performed, the plasmid DNA was cleaved with *NheI* and *XhoI* to confirm the presence of inserts in the correct orientation in the pET-28b(+) vector. Plasmid DNA in the correction orientation resulted in a restriction profile consisting of two fragments of ~5300 bp and ~770bp, representing the pET-28b(+) backbone and the inserted DNA, respectively (Figure 4.5).

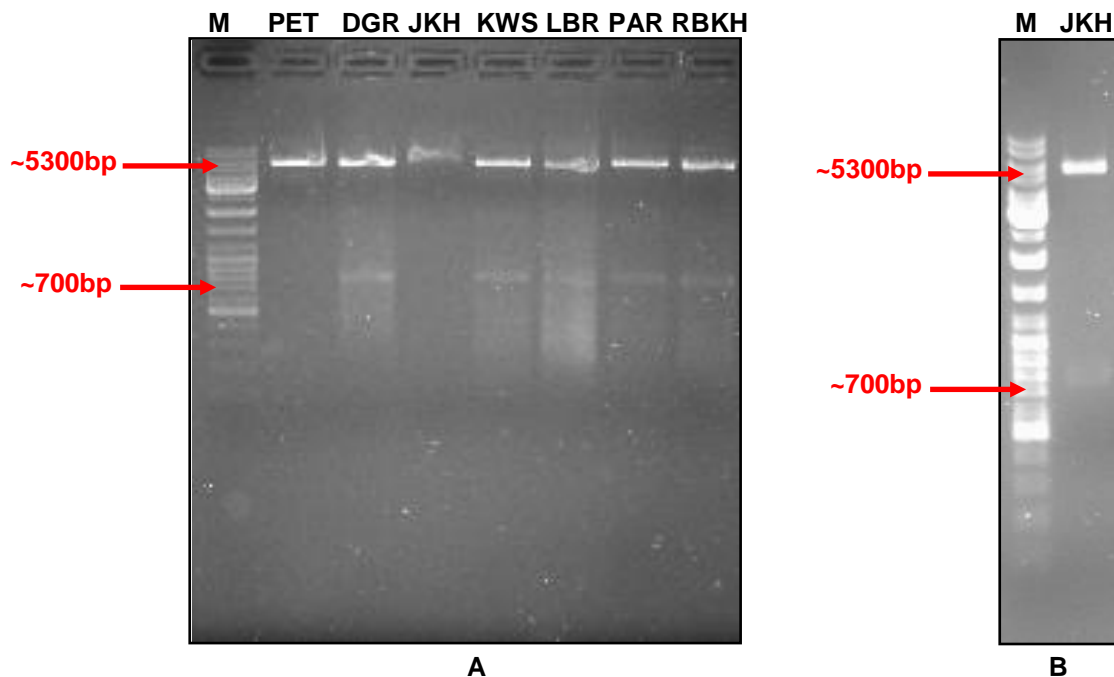


Figure 4.5: 1% w/v agarose gel visualised under UV illumination showing the restriction profile of the digested plasmid DNA isolated from TOP 10 competent *E. coli* when cleaved with *NheI* and *XhoI*. **A:** The lane indicated as PET is the pET-28b(+) plasmid with no insert; all the lanes show the presence of insert DNA in the vector, with the exception of sample JKH. **B:** Plasmid DNA from sample JKH re-digested indicates the presence of insert DNA. Lanes M represent the O'GeneRuler™ DNA Ladder Mix (Fermentas) molecular marker.

The positive clones were sequenced with the T7 promotor and terminator primers before commencing with protein expression to insure that the DNA was inserted into the pET-28b(+) vector correctly and in frame.

4.3.3. SEQUENCING RESULTS OF RECOMBINANT PLASMID DNA

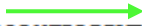
The sequence data obtained was assembled with *Vector NTI Advance™ 9.0* software and aligned with *DNAssist 3.0*. Alignments of the plasmid DNA with the sequence data obtained in Chapter 3 revealed that five of the isolates cloned into pET-28b(+) were inserted correctly within frame (data not shown). The sequence alignment of isolate JKH in the plasmid with the sequence data obtained in Chapter 3 revealed that there were deletions of two bases within the sequence (Figure 4.6).

JKH	1			0
PET28B_JKH	1	ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCCGCGCAAGCCATAT		62
		→		
JKH	1	CGCTAGCCTGTGGGGCACCTCTAACTGCGCATGCGCTATATTTTCAGATTAGACGACGTAGAT		62
PET28B_JKH	63	GGCTAGCCTGTGGGGCACCTCTAACTGCGCA GCGCTATATTTTCAGATTAGACGACGTAGAT		123
JKH	63	ATGCCCCGCCATACTACCGCAGACGTCACAACAGGCGATACCGCCGAAGACGTAGATACTTC		124
PET28B_JKH	124	ATGCCCCGCCATACTACCGCAGACGTCACAACAGGCGATACCGCCGAAGAC TAGATACTTC		184
JKH	125	CGCAGACGCCGTTTCTCAACCAATAGAATTTACTACTATCAGATTCAAACGCCAATTCAAATT		186
PET28B_JKH	185	CGCAGACGCCGTTTCTCAACCAATAGAATTTACTACTATCAGATTCAAACGCCAATTCAAATT		246
JKH	187	CGAGATTGAAAGCAAACCACCAGTACTGGCCAAGTAATTTGGAATTCTGACTACATAACCT		248
PET28B_JKH	247	CGAGATTGAAAGCAAACCACCAGTACTGGCCAAGTAATTTGGAATTCTGACTACATAACCT		308
JKH	249	TTGCATTGTCAGACTTCCTAGCAACAGCGCCAAACCCACATACTAAATTTTGAAGACTAC		310
PET28B_JKH	309	TTGCATTGTCAGACTTCCTAGCAACAGCGCCAAACCCACATACTAAATTTTGAAGACTAC		370
JKH	311	CGGATTAAATTAGCTAAAATGGAATTGAGGCCACATGGGGCCACTACACAATTAGTGCAGA		372
PET28B_JKH	371	CGGATTAAATTAGCTAAAATGGAATTGAGGCCACATGGGGCCACTACACAATTAGTGCAGA		432
JKH	373	CGGATTTCGGCCACACGGCCATAATCCAAGATTCCAGAATAACTAAATTTAAGACTACTACAG		434
PET28B_JKH	433	CGGATTTCGGCCACACGGCCATAATCCAAGATTCCAGAATAACTAAATTTAAGACTACTACAG		494
JKH	435	ACCAAACCTCAAGACCCAGTGGCTCCTTTTCGACGGTGCAAAAAAGTGGTATGTTGCCAGGGGA		496
PET28B_JKH	495	ACCAAACCTCAAGACCCAGTGGCTCCTTTTCGACGGTGCAAAAAAGTGGTATGTTGCCAGGGGA		556
JKH	497	TTCAAACGCCTCCTCAGACCGAAACCTCAAATAACTATAGATGACCTGACCACTGCGAACCA		558
PET28B_JKH	557	TTCAAACGCCTCCTCAGACCGAAACCTCAAATAACTATAGATGACCTGACCACTGCGAACCA		618
JKH	559	GTCTGCGGCATTGTGGCTAAACAGCGCCAGAACCGGCTGGATCCCCTACAAGGAGGACCCA		620
PET28B_JKH	619	GTCTGCGGCATTGTGGCTAAACAGCGCCAGAACCGGCTGGATCCCCTACAAGGAGGACCCA		680
JKH	621	ATGCGGCAGGAGCGAACGTCAAGCACTACGGCCTTGCATTTCAGCTTCCCTCAGCCGGAAGTG		682
PET28B_JKH	681	ATGCGGCAGGAGCGAACGTCAAGCACTACGGCCTTGCATTTCAGCTTCCCTCAGCCGGAAGTG		742
JKH	683	ACAATCACTTATGTATGCGAGCTCACTCTGTATGTTCAATTCCGTGAGTTTGCCCCCAACAA		744
PET28B_JKH	743	ACAATCACTTATGTATGCGAGCTCACTCTGTATGTTCAATTCCGTGAGTTTGCCCCCAACAA		804
		←		
JKH	745	TCCAGTACTTAACTCGAG	763	
PET28B_JKH	805	TCCAGTACTTAACTCGAGCACCACCAC	832	

Figure 4.6: Sequence alignment of isolate JKH as sequenced in Chapter 3 and the cloned product within the pET-28b(+) vector. The forward arrows indicate the start of the CP gene and the reverse arrow the end. Two deletions were observed within the cloned product and are indicated in green.

It is possible that the deletions of the nucleotides within the CP gene of isolate JKH could have occurred during PCR amplification of the gene where the correct bases weren't incorporated during extension of the template DNA.

The CP gene of isolate JKH in pET-28b(+) was translated with Vector *NTI Advance*[™] 9.0 software. The deletions of the two nucleotides caused the reading frame of the codons to shift, which in turn resulted in the occurrence of stop codons (TAA, TAG and TGA) throughout the CP gene sequence of isolate JKH (Figure 4.7).



LWGTSNCAALYFRLDDVDMPAHTTADVTTGDTAED*ILPQTPFLNQ*NLHYQIQTPIQIRDSKANHQYWPSNLEF*LHNLICIVRLP
 SNSAKPTYTKFRRLPD*IS*NGIEAHMGPLHN*CRRIRPHGHNPRFQNN*I*DYYRPNRSPSGSFRRCCKVCCQGIQTTPQTETS
 NNYR*PDHCEPVCGIVAKQRQNRDPTTRRTQCGRSERQALRPCIQLPASGSDNHLCMRAHSVCSIPSVCPQQSQYL

Figure 4.7: The translated sequence of isolate JKH showed that a frame shift occurred from the 9th amino acid (the start of the frame shift is indicated by the green arrow). The frame shift resulted in the generation of stop codons through out the amino acid sequence (indicated with the red stars).

Due to the occurrence of stop codons in the sequence of isolate JKH, this isolate was omitted from further expression studies, as these codons would prevent the expression of the protein.

4.3.4. EXPRESSION OF THE RECOMBINANT COAT PROTEIN GENES

Small scale plasmid isolations were performed on the remaining five isolate (DGR, KWS, LBR, RBKH and PAR) to remove the plasmid DNA from the TOP 10 *E. coli* cells. The plasmid DNA was successfully transformed into the BL21(DE3) *E. coli* host strain for subsequent protein expression.

4.3.4.1. PROTEIN EXPRESSION WITH 0.4 mM IPTG

Five isolates were used in protein expression studies. BL21(DE3) *E. coli* cells containing the recombinant plasmid DNA was grown until OD₆₀₀ = 0.6 – 1.0 and induced with a final concentration of 0.4 mM IPTG. Expression was performed at three different temperatures: 19°C O/N, 30°C for 8 hours and 37 °C for 5 hours. Pre-induction (t₀) samples were collected as well as post induction. These samples were analysed on SDS-PAGE gels. The expected band sizes for the expressed CPs was ~30 kDa.

One of the limitations of the T7 promoter systems is that the target protein is often unable to reach native conformation and either partially or completely segregates within inclusion bodies (Baneyx, 1999), thus inclusion body purifications were also performed on the collected fractions with the B-PER[®] Bacterial Protein Extraction Reagent.

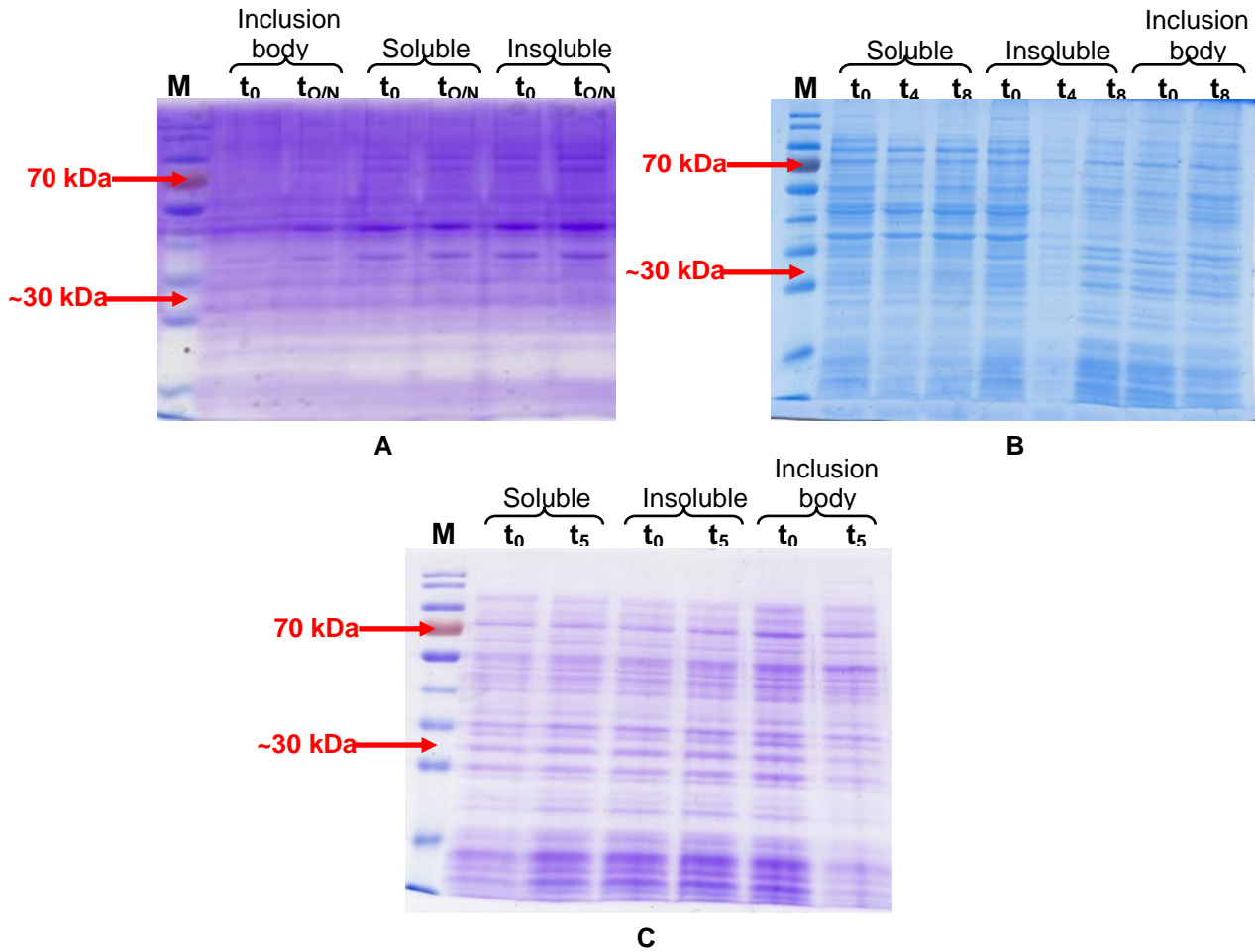


Figure 4.8: 10% SDS-PAGE gels stained using the Fairbanks Method (Fairbanks *et al.*, 1971 [A and C]) and PageBlue[™] Protein Staining Solution (Fermentas [B]). All the *E. coli* cells were induced with a final concentration of 0.4 mM IPTG, but incubated at varying temperatures and times. No bands (~30 kDa) indicating heterologous protein expression in either of the soluble and insoluble fractions as well as the inclusion body fraction was found at 19°C O/N (A), 30°C for 8 hours (B) or 37°C for 5 hours (C). Lane M represents the PageRuler[™] Prestained Protein Ladder (Fermentas).

No heterologously expressed protein bands were detected post-induction for the soluble, insoluble and inclusion body fractions. However, there were bands at ~ 30 kDa in all the lanes for expression at the three different temperature ranges, but they did not increase in intensity at all which suggested that possibly no expression of the target protein occurred, or that expression was not under complete control of IPTG. The gels shown in Figure 4.8 represent the expression profiles obtained for isolate DGR at the various temperatures with a final IPTG induction concentration of 0.4 mM. Expression of the other four isolates (KWS, LBR, RBKH and PAR) revealed the same results as was observed for DGR (data not shown).

4.3.4.2. PROTEIN EXPRESSION WITH 1 mM IPTG

It was thought that perhaps protein expression was not under the complete control of IPTG, as protein bands were observed at the expected size (~ 30 kDa) of the CPs to be expressed in both the pre-induction and post-induction fractions at 0.4 mM IPTG, thus the IPTG concentration was adjusted to 1 mM.

The study was performed at the following temperature parameters: 19°C O/N, 30°C for 8 hours and 37°C for 5 hours. Pre-induction (T_0) as well as post-induction samples were taken to determine whether the proteins were expressed using the higher concentration of IPTG to induce the *E. coli* cells.

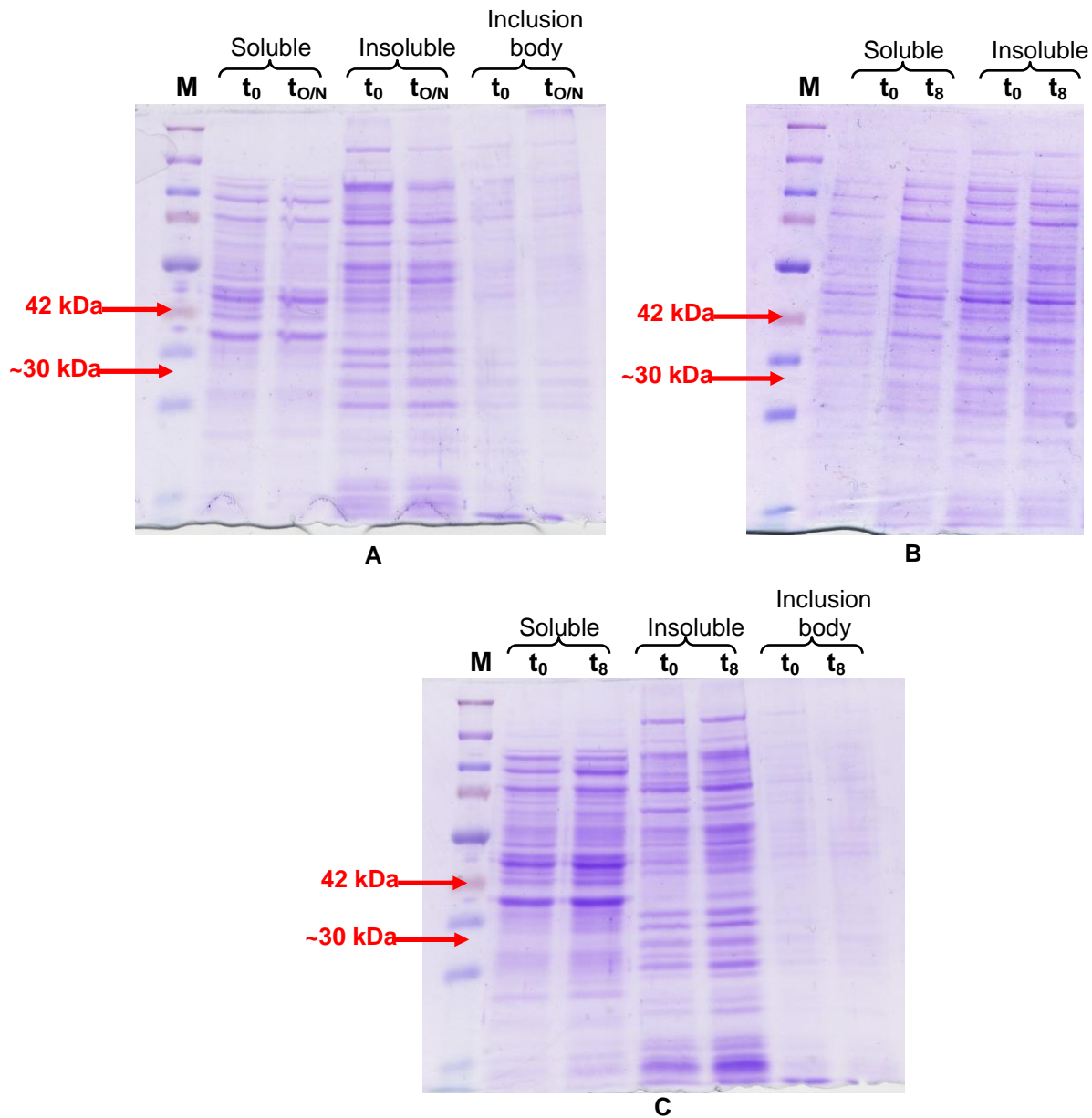


Figure 4.9: 10% SDS-PAGE gels stained using the Fairbanks Method (Fairbanks *et al.*, 1971). The *E. coli* cells were induced with a final concentration of 1 mM IPTG, but incubated at different temperatures and times. No bands indicating heterologous protein expression in either of the soluble and insoluble fractions as well as the inclusion body fraction was found at 19°C O/N (A) and 37°C for 5 hours (C). No heterologously expressed protein was detected in the soluble and insoluble fractions at a temperature of 30°C for 8 hours; the inclusion body fraction is omitted from this gel (B). Lane M represents the Spectra™ Broad Range Protein Ladder (Fermentas).

The SDS-PAGE gels revealed the same expression profile as was observed at expression with 0.4 mM IPTG. From these results it could not be conclusively established if there was some or no protein expression with SDS-PAGE, as bands were observed at ~30 kDa for both pre- and post-induction samples.

Western blots were performed with the SuperSignal[®] West HisProbe[™] KIT on the samples in order to detect the expressed proteins with chemiluminescence. No chemiluminescent bands were observed on Western blots (data not shown). No expression of recombinant protein occurred during any of the expression studies performed, the bands observed on the SDS-PAGE gels were native *E. coli* proteins that were extracted with the B-PER[®] Bacterial Protein Extraction Reagent (PIERCE).

It was deemed a possibility that the recombinant proteins were expressed at extremely low levels and the expressions were scaled up to 1 L, but again SDS-PAGE analysis and Western blotting of the samples revealed that none of the desired proteins were expressed.

Due to the lack of expression of recombinant full length CP for any of the isolates, the expression system was evaluated further by means of looking at the codon compatibility between *E. coli* and the CP genes that were unsuccessfully expressed.

The genetic code is degenerate, meaning there are multiple codons that code for the same amino acid. Each organism employs usage of these codons differently (Ikemura, 1985). GC-rich organisms prefer GC-containing codons over AT-containing ones, thus there are optimal and less optimal codons for each organism. The codon “language” is not universal and is unique for each organism and the codon usage of various genes of one organism relates to their rate of expression (Gouy and Gaultier, 1982; Grote *et al.*, 2005). In molecular biology, codon usage has an impact on the heterologous expression of genes. If a target gene contains codons that are rarely used by the host organism, it can lead to poorly translated messenger ribonucleic acids (mRNAs), decreased mRNA stability and at times premature termination of translation and

inhibition of cell growth (Sorensen *et al.*, 1989). The usage of rare codons for Arginine (Arg) in *E. coli* can also cause the misincorporation of lysine (Lys) for Arg as was found by Calderone and co-workers (1996).

The N-terminus of the CPs used in this study contained an abundance of Arg residues, in the form of the codons AGA (occurring eight times) and AGG (occurring once). The presence of these residues at the N-terminus of the gene could be the reason why no expression was achieved in *E. coli* cells during this study. According to Sorensen and Mortensen (2005), transcripts containing rare codons in clusters (doublets or triplets) accumulate in large quantities, causing transcriptional errors to arise from rare codon bias. This is possibly the case for the BFDV CP as there are three clusters of Arg rich areas that constitute the potential bipartite nuclear localization signals (NLSs) as described by Heath *et al.*, 2006 (Figure 4.10).

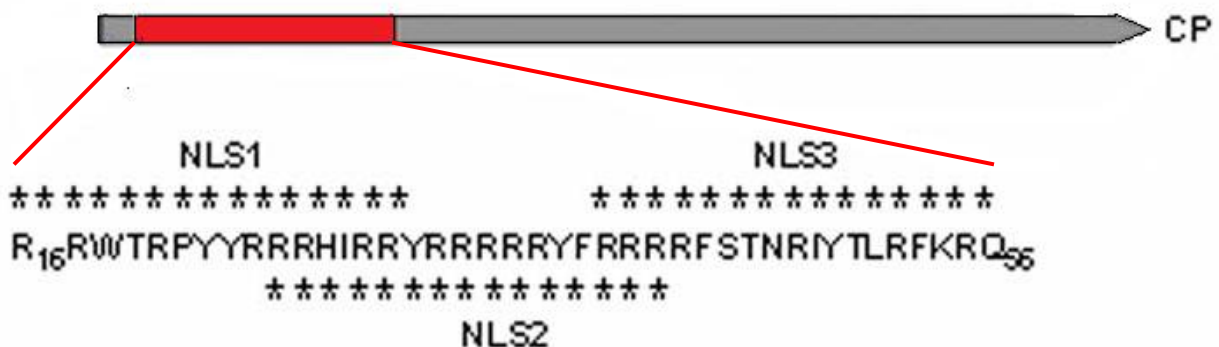


Figure 4.10: A schematic map adapted from Heath *et al.*, 2006 indicating the section of the CP that contain the putative bipartite NLSs in red, the sequence shows the three clusters abundant with Arg residues.

The codon adaptation index (CAI) evaluates how well a gene is adapted to the translational machinery of the host organism. The CAI is a single value measurement that summarizes codon usage of a gene relative to the codon usage of a reference set of genes (Sharp and Li, 1987). A CAI of “one” suggests that the gene of interest is likely to be expressed in high concentrations, whereas a value of “zero” indicates no expression at all. The CAI was calculated for the full length CP gene of BFDV at the following web server: <http://cbi.labri.fr/outils/Pise/cai.html>. The calculation was

performed in order to determine what the likelihood was that these genes would be expressed in *E. coli*. The CAI was calculated to be 0.250, which indicated that there was a 25% chance that the CP gene would be expressed in *E. coli*. It can be suggested from this study that calculation of CAI should be the first procedure to be performed before undertaking any expression in *E. coli*.

Johne and co-workers (2004) failed to express the full length CP gene in *E. coli*; they reported that it was due to the accumulation of Arg residues at the N-terminal of the CP. They did achieve expression of a truncated version of the protein where 38 amino acids were deleted from the N-terminus of the protein. Kondiah (2008) achieved expression of the full length recombinant CP in the pBAD/His B bacterial expression system utilising TOP 10 *E. coli* as expression host, albeit not at very high concentrations.

There are two ways with which to circumvent the problems incurred in the *E. coli* expression system. The first is to use *E. coli* strains such as the BL21-CodonPlus[®] strains from Stratagene which are engineered to contain extra copies of the genes that encode the tRNAs that limit translation of heterologous proteins in *E. coli*, this will allow high-level expression of recombinant genes that are poorly expressed in conventional *E. coli* strains. The second method would be to adapt the codon usage of the gene to be expressed in the host organism, such as truncating the protein in order to remove the codons that are rarely used by the host. This approach would not be suitable for the investigation of possible antigenic diversity of the CP (which was the aim of the study) as it was desirable to express the full length CP genes in order to investigate whether any genetic variation that occurred would lead to antigenic variation of the protein.

Expression of the full length recombinant CP was successfully achieved on more than one occasion by independent researchers (Heath *et al.*, 2006 and Stewart *et al.*, 2007) in a baculovirus expression system utilising *Spodoptera frugiperda* insect cells as expression host. It would seem that use of the baculovirus expression system for the full length CP is more reliable than expression of this gene in *E. coli*.

4.4. CONCLUSIONS

There is a need to express the antigenic protein of BFDV in high volumes in order to establish reliable serological tests, such as ELISAs, with which to diagnose disease in birds rapidly as well as to determine the titer of antibodies present in birds. However, before any test can be 100% effective, it is important to determine whether there are indeed antigenic differences within the CP gene of BFDV, as this is the antigenic protein of the virus and is used both in the development of diagnostic tests as well as the development of vaccines.

It was the aim of this study to recombinantly express the CP gene in a bacterial expression system. The expressed proteins would be used in an ELISA to determine whether there were antigenic differences between six different isolates of the CP gene of BFDV. Unfortunately, our expression system proved to be very ineffective as no expression of the desired genes was achieved. From this study it can be concluded that utilizing *E. coli* to express the full length CP gene of BFDV is not a viable option. Due to the fact that the CP gene contains codons that are rarely used by *E. coli*, it will always hamper expression of the CP gene, unless engineered strains of *E. coli* are used that can efficiently translate the rare codons.

Therefore, it is suggested that future research with recombinant protein should be done in an eukaryotic expression system such as the baculovirus expression system which has been shown by different authors to express the entire CP gene successfully. Once the full length gene is expressed, the protein can be used to determine whether genetic differences within the CP gene will relate to antigenic variation.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

Psittacine beak and feather disease (PBFD) is a dermatological condition of psittacine species caused by *Beak and feather disease virus* (BFDV). PBFD was first recognized in 1975 by Dr. Ross Perry in Australia. At present the disease is known to affect more than 40 species of both Old and New World psittaciformes on all continents. According to the Australian Commonwealth government PBFD is a key threatening process to the survival of five endangered species.

In South Africa bird breeders lose 10 – 20% of breeding stocks due to PBFD each year (Heath *et al.*, 2004). According to Kondiah (2008) the value of the loss incurred by breeders for their breeding stocks is approximately R 4 000 000 each year. Although the monetary losses incurred are a tremendous problem amongst breeders in South Africa, the bigger issue at hand is the fact that PBFD severely threatens the survival of the indigenous endangered Cape parrot (*Poicephalus robustus*). In Zambia the vulnerable black-cheeked lovebird (*Agapornis nigrigenis*) is also endangered with extinction due to the incidence of PBFD.

PBFD is caused by BFDV, which is an environmentally stable circovirus which is not affected by disinfection procedures. PBFD is a fatal disease for which there is no preventative vaccine or cure available. Treatment of diseased birds is only supportive. When birds under the age of three years are infected with BFDV, they are most likely to succumb to secondary bacterial and fungal infections as a result of immunosuppression caused by BFDV targeting, and replicating in the bursa of Fabricius. Normally, older birds that are infected with BFDV become carriers of the disease and are responsible for both the horizontal and vertical transmission of the virus to other susceptible birds.

Attempts to cultivate the virus in tissue/cell cultures and embryonated eggs have not been successful; this contributes to the lack of effective control measures against the disease, the development of reliable standardized serological tests and vaccine development.

Numerous studies have been conducted on the genetics of the virus in order to find alternative routes with which diagnostic tests and vaccines can be developed. During these studies it has been shown that BFDV is a genetically diverse virus (Niagro *et al.*, 1998; Bassami *et al.*, 2001; Raue *et al.*, 2003; Heath *et al.*, 2004). Heath and co-workers (2004) showed that BFDV isolates clustered into eight genotypes (lineages) on a phylogenetic tree. The researchers found that the diversity in Southern African isolates is similar to that found in Australia and New Zealand. They also found that Southern African isolates clustered into three unique lineages and this suggested the possibility that BFDV was introduced into Africa on three different occasions. Ritchie and co-workers (2003) suggested that all variants of BFDV might infect all psittacine species but only certain genotypes are pathogenic to a certain species group.

Even though so much research has been performed on the genetics of the virus, no studies have been conducted to relate these genetic differences to the antigenicity and pathogenicity of BFDV. However, in 2008 Shearer and co-workers suggested the presence of an antigenically distinct BFDV that is adapted to infecting cockatiels. Currently, researchers are developing vaccines and serological tests under the assumption that there is only one strain of BFDV that infects psittacine birds.

The primary aim of this study was to investigate the antigenicity of genetically different isolates of BFDV. This was done in order to establish whether or not genetic variation of the virus can lead to the existence of different strains of BFDV. As the coat protein (CP) of BFDV is suggested to be the epitopic protein, it was to be used for antigenic analysis and expression experiments performed in this study.

Six BFDV isolates were obtained from different geographical locations as well as different psittacine species in South Africa. The entire CP genes of these isolates were amplified by polymerase chain reaction (PCR) and sequenced. The aligned sequence data of the six isolates showed that there were many nucleotide substitutions throughout the sequences. The amino acid alignments of the sequence data indicated a high degree of identity to each other, but there were several substitutions within the sequences. Isolates JKH and RBKH were shown to have the exact same amino acid sequences, this was expected as both were isolated from the same farm in KwaZulu Natal.

A neighbour-joining phylogenetic tree was constructed with the amino acid sequence data from the isolates used in this study, as well as from isolates described by Heath *et al.*, 2004. Phylogenetic analysis indicated that the six isolates grouped into the first lineage as described by Heath and co-workers (2004).

The accuracy of secondary structure prediction algorithms was analyzed; these predictions would be helpful in predicting where β -bends occurred on the CP. Analysis of the programs with three different proteins of known crystal structure showed that secondary structure predictions still have a long way to go to accurately predict where secondary features occur within the protein. The CP of BFDV has no homologs in the Protein Data Bank (PDB), which makes accurate homology modelling of the structure of the CP impossible.

In silico antigenic predictions of the isolates from this study suggest that both Australian and South African isolates displayed the same antigenic profiles. Isolates LK-VIC from Australia and BCL1-ZAM from Zambia each form their own lineage on the phylogenetic tree – this trend was also observed with their antigenic predictions as each of these isolates presented its own antigenic profile.

The *in silico* data obtained indicated that there was a distinct possibility for the existence of different strains of BFDV, although a certain genetic pattern contributing to

antigenicity could not be determined. The analysis showed that one antigenic determinant for both the South African and Australian isolates were constantly detected at the N-terminus of the CP; this indicated that the CP could not be truncated in order to study antigenicity of the protein *in vitro*.

Further, it was undertaken to express the full length CP genes of the six isolates described in this study in a *Escherichia coli* expression system using the pET-28b(+) vector system from Novagen.

After numerous attempts at heterologously expressing the CP gene at varying concentrations of IPTG, different temperatures and incubation periods, the expression was found to be unsuccessful as no recombinant protein was detected with sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) as well as Western blot analysis. The codon adaptation index (CAI) for *E. coli* with the CP was determined to be 0.250, thus indicating that the full length CP gene had a 25% possibility of being successfully expressed. The reason for the low CAI was due to the fact that the codons encoding the Arginine residues at the N-terminus of the CP were not compatible for efficient translation by *E. coli*. From the findings in this study it can be suggested that before attempting heterologous expression of any gene in *E. coli* the CAI should be calculated in order to ascertain whether the amino acids in the gene is compatible with the codon usage of *E. coli*.

From this work it is concluded that no further attempts to express the full length CP gene in *E. coli* should be conducted. For future expression studies with the CP gene of BFDV, it is suggested to use the baculovirus expression system as it has been shown by independent researchers (Heath *et al.*, 2006; Stewart *et al.*, 2007) to successfully express the entire CP gene.

Based on the results obtained in the *in silico* antigenic predictions it is imperative to express the full length CP gene as there is a possibility that at least one antigenic determinant is situated at the N-terminus of the protein. *In silico* predictions of

antigenicity can be a helpful tool with which to hypothesize where possible antigenic determinants of a protein can be. These determinants can then be selected for further *in vitro* studies of their possible antigenic properties.

It is of importance to confirm or reject the existence of more than one strain of BFDV. This will affect both the development of reliable diagnostic tests as well as development of a vaccine as preventative measure against PBFD.

SUMMARY

Psittacine beak and feather disease (PBFD) is a dermatological condition that affects both captive and wild psittacine birds worldwide. In Southern Africa, 10 – 20% of breeding stocks are lost due to the disease each year. PBFD threatens the survival of the indigenous endangered Cape parrot (*Poicephalus robustus*) as well as the black-cheeked lovebird (*Agapornis nigrigenis*).

The disease is characterized by roughly symmetrical feather loss, feather abnormalities, anorexia and immunosuppression. In advanced cases of the disease beak and claw deformities are present. The causative agent of PBFD is *Beak and feather disease virus* (BFDV), a circovirus belonging to a diverse group of circoviruses within the family *Circoviridae*. BFDV has a circular single stranded DNA genome consisting of seven open reading frames (ORFs); three of these ORFs are conserved amongst all isolates of BFDV. ORF 1 encodes the Rep protein, ORF 2 the coat protein (CP) which is also the epitopic protein of the virus and ORF 5, whose function remains unclear.

BFDV cannot be cultivated in tissue/cell culture or in embryonated eggs. The inability to cultivate the virus has hampered the development of diagnostic tests and a vaccine as preventative measure against the disease.

BFDV is a genetically diverse virus. Researchers have demonstrated that there are at least eight different lineages of BFDV, where Southern African isolates group into three unique genotypes. Many studies have been performed which indicated the diversity of BFDV, but so far no studies have been done which link this genetic diversity to the possibility of the existence of more than one strain of BFDV. This led to the aims of the present study which were to investigate antigenicity of BFDV isolates belonging to

different genotypes and then the subsequent bacterial expression of six isolates of BFDV that were genetically different.

The entire CP genes of six isolates were amplified with polymerase chain reaction (PCR) and subsequently sequenced. Phylogenetic analysis of sequence data showed that the isolates from this study grouped into lineage one as was described by Heath and co-workers (2004). Amino acid sequences from the isolates from each lineage was applied in an *in silico* prediction algorithm in order to establish the possibility of more than one strain of BFDV. The predictions indicated that isolates from Australia and South Africa had the same antigenic profile. However, isolate BCL1-ZAM and LK-VIC each produced their own antigenic profile. This indicated the distinct possibility that there is more than one strain of BFDV and that at least one antigenic determinant was situated at the N-terminus of the CP. However, these results have to be confirmed by conducting *in vitro* studies.

Attempts were made to express the full length CP genes of six isolates in BL21(DE3) *Escherichia coli* with the pET-28b(+) vector. Neither polyacrylamide gel electrophoresis (PAGE) nor Western blotting indicated the presence of recombinantly expressed protein in any of the studies conducted. The codon adaptation index (CAI) for BFDV was calculated to be 0.250, which indicated that the CP had a 25% possibility of being expressed in *E. coli* due to codon incompatibility. From this study it can be suggested that before attempting expression of any gene in *E. coli* the CAI should be calculated. It was also concluded from this work that no further attempts to express the CP gene in *E. coli* should be conducted.

Keywords: Psittacine beak and feather disease (PBFD), Beak and feather disease virus (BFDV), *in silico*, strains, bacterial expression, codon adaptation index (CAI).

OPSOMMING

Psittacine beak and feather disease (Pbfd) is 'n dermatologiese toestand wat beide gevange en wilde papagaaië wêreldwyd affekteer. In Suidelike Afrika is daar 'n verlies van 10 – 20% van die broeivoëls elke jaar. Pbfd bedreig die oorlewing van die alreeds bedreigde inheemse Kaapse papagaai (*Poicephalus robustus*) sowel as die black-cheeked lovebird (*Agapornis nigrigenis*).

Pbfd word gekarakteriseer deur die simmetriese verlies van vere, veerabnormaliteite, anorexia en immuunonderdrukking. In gevorde gevalle van die siekte vertoon voëls bek en klou abnormaliteite. Pbfd word veroorsaak deur die *Beak and feather disease virus* (BFDV), 'n circovirus wat behoort aan 'n diverse groep van circovirusse geklassifiseer in die familie *Circoviridae*. BFDV het 'n sirkulêre enkelstring DNA genoom wat bestaan uit sewe ooplesrame (OLRe); drie van die OLRe is gekonserveerd in alle BFDV isolate. OLR 1 kodeer vir die Rep proteïen, OLR 2 vir die kapsied proteïen (KP) wat ook die epitopiese proteïen van die virus is en OLR 5 kodeer vir 'n proteïen waarvan die funksie onbekend is.

BFDV kan nie in weefselkulture of geëmbriioneerde eiers gekweek word nie. Die onvermoë om die virus te kultiveer vermoeilik die ontwikkeling van diagnostiese toetse asook die ontwikkeling van 'n entmiddel as voorkoming teen die siekte.

BFDV is 'n geneties diverse virus. Navorsers het gedemonstreer dat daar ten minste agt verskillende genotipes van BFDV is. Suid Afrikaanse isolate groepeer in drie unieke genotipes. Veelvuldige studies is gedoen wat die diversiteit van BFDV aandui, maar tot dusver is geen studies gedoen wat hierdie genetiese diversiteit koppel aan die moontlikheid van die bestaan van meer as een infektiewe stam van BFDV. Hierdie het gelei tot die doel van die huidige studie wat ingesluit het navorsing in die antigenisiteit

van BFDV isolate wat behoort aan verskillende genotipes asook die bakteriese uitdrukking van ses isolate van BFDV wat geneties verskil het.

Die volledige KP geen van ses BFDV isolate is geamplifiseer deur polimerase ketting reaksie (PKR) en daarna is die basis paar opeenvolging van die produkte bepaal. Filogenetiese analise van die basis paar opeenvolging data het gewys dat die isolate in die studie gebruik gegroep het in die eerste genotipe soos beskryf deur Heath en mede-werkers (2004). Aminosuur opeenvolgings van die isolate is gebruik in 'n *in silico* voorspellings algoritme om te bepaal of daar meer as een stam van BFDV is. Die voorspellings het aangedui dat isolate vanaf Australia en Suid Afrika dieselfde antigeniese profiele gehad het. Die isolate BCL1-ZAM en LK-VIC het elkeen hul eie antigeniese profiel gehad. Die resultate het gedui op die moontlikheid dat daar meer as een infektiewe stam van BFDV is en dat ten minste een antigeniese determinant aan die N-terminus van die KP geleë is. Hierdie bevindings moet bevestig word deur *in vitro* studies uit te voer.

Daar is gepoog om die vollengte KP geen van ses isolate uit te druk in BL21(DE3) *Escherichia coli* met die pET-28b(+) vektor. Nie poli-akrielamied gel elektroforese (PAGE) of Western blotting het aangedui dat rekombinante proteïen tydens die eksperimente uitgedruk was nie. Die kodon aanpassings indeks (KAI) vir BFDV was bereken om 0.250 te wees, wat aangedui het dat die KP 'n 25% kans gehad het om in *E. coli* uitgedruk te word as gevolg van kodon onversoenbaarheid. Vanuit hierdie studie kan dit voorgestel word dat die CAI bereken moet word voordat uitdrukking van enige geen in *E. coli* uitgevoer word. Vanaf hierdie studie kan ook bepaal word dat verdere studies om die KP in *E. coli* uit te druk gestaak moet word.

Sleutelwoorde: Psittacine beak and feather disease (PBFD), *Beak and feather disease virus* (BFDV), *in silico*, stam, bakteriese uitdrukking, kodon aanpassings indeks (KAI).

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