Development of a DNA Vaccine for the Prevention of Psittacine Beak and Feather Disease.

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For my father, Mr. Mohamedali Tajbhai, the inspiration behind this

degree

For Lloyd who is my beacon of light in times of darkness

And

To my mother, Mrs. Wijitha Tajbhai and the rest of my family whose

love, pride and faith in me never waver.

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ACKNOWLEDGEMENTS.

CHAPTER 1: PSITTACINE BEAK AND FEATHER DISEASE AND DNA VACCINES: A REVIEW.

1.1. INTRODUCTION.

Circoviruses are animal viruses with a circular single stranded DNA (ss-DNA) genome which is covalently closed and are classified in the family *Circoviridae*. They are small, non-enveloped spherical viruses (diameter 15-25 nm) associated with potentially fatal disease in their respective hosts. Members of *Circoviridae* are classified into the genus *Gyrovirus* or *Circovirus*.

Porcine circovirus type 1 (PCV-1) was first demonstrated in Germany in 1974 as a contaminant of cultured pig kidney cells and forms the type member of the genus *Circovirus* (Studdert, 1993). Viruses that also belong to this genus are *Beak and feather disease virus* (BFDV), *Canary circovirus* (CaCV), *Porcine circovirus type 2* (PCV-2), *Pigeon circovirus* (PiCV) and *Goose circovirus* (GoCV) (Todd *et al.*, 2001). Tentative members placed in the genus are *Duck circovirus* (DuCV), *Columbid circovirus* (CoCV) and *Bovine circovirus*. *Chicken anaemia virus* (CAV) is the only member of the genus *Gyrovirus* and causes a disease in young chickens characterized by anaemia, lymphoid depletion and haemorrhaging leading to increased mortality (Todd, 2000).

The following review, although not exhaustive on the various aspects discussed, consists of information on Psittacine beak and feather disease (PBFD), the importance of immunity (commonly suppressed in PBFD positive birds) and the possibility of using DNA vaccines to prevent outbreaks of PBFD.

1.2. PSITTACINE BEAK AND FEATHER DISEASE (PBFD).

Psittacine beak and feather disease (PBFD) was first described in cockatoos in Australia by Dr. Ross Perry in 1975 (Heath *et al.*, 2004). It is characterised by varying degrees of symmetric feather dystrophy and loss and is caused by BFDV. PBFD outbreaks of wild populations are commonly found in Australia while outbreaks of captive birds occur worldwide.

1.2.1. <u>VIRUS ASPECTS.</u>

1.2.1.1. Biochemical and Physical Characteristics.

BFDV has an icosahedral, non-enveloped virion between 14-17 nanometres (nm) in diameter [Figure 1.2.1] (Ritchie *et al.*, 1989). It is smaller than CAV and lacks the distinctive surface structure CAV exhibits. BFDV is reported to share more similarities with PCV (Todd *et al.*, 1991). Due to the similarities in ultrastructure and DNA composition to CAV and PCV, BFDV is suggested to be highly environmentally stable. Stability tests cannot be performed due to the inability to grow the virus in cell culture.
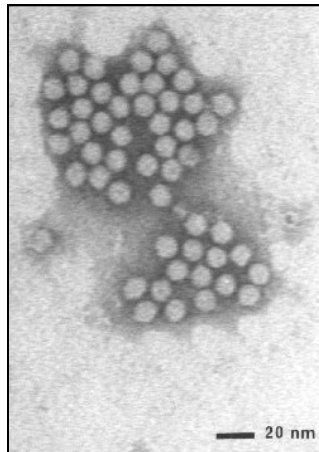
Figure 1.2.1: An electron micrograph of negatively stained *Beak and feather disease virus* particles (http://numbat.murdoch.edu.au/caf/pbfd.htm).

The inability to propagate BFDV *in vitro* led to the purification of the virus from infected feather pulp in order to study its physical characteristics as well as for use in the development of a haemagglutination assay (HA) and haemagglutination inhibition assay (HI) for the detection of viral antigen and antibody, respectively as BFDV has agglutinating activity with erythrocytes from a range of psittacine species. Polyacrylamide gel electrophoresis (PAGE) revealed the expression of three major viral associated proteins [Table 1.1] with molecular weights (Mr) of 26.3 kiloDaltons (kDa), 23.7 kDa and 15.9 kDa (Todd *et al.*, 1991).

Electron microscopy revealed the presence of intracytoplasmic inclusion bodies appearing as paracrystalline arrays, semicircles, concentric circles, whorls and other configuration consisting of electron-dense granules 17-22 nm in diameter.

Table 1.1: Biochemical and physical characteristics of *Chicken anaemia virus*, *Porcine circovirus type 1* and *Beak and feather disease virus* (Adapted from Todd, 2000).

	CAV	PCV-1	BFDV
Particle size (nm)	19.1-26.5	16.8-20.7	14-20.7
Genome size	2298/2319	1759	1993-2018
Virion proteins (M_r)	50 000	36 000	26 300, 23 700, 15 900

1.2.1.2.   Genome Organisation.

The ambisense genome is composed of ss-DNA arranged into seven open reading frames (ORFs) identified by Bassami and co-workers (2001) although not all isolates of BFDV have all seven ORFs present in their genomes. Three ORFs are located on the virus (V) sense strand and four on the complementary (C) sense strand [Figure 1.2.2]. ORFs 1, 2 and 5 are conserved among all isolates and encode a replication associated protein (Rep), coat protein (CP) and a protein whose function is as yet unknown, respectively.
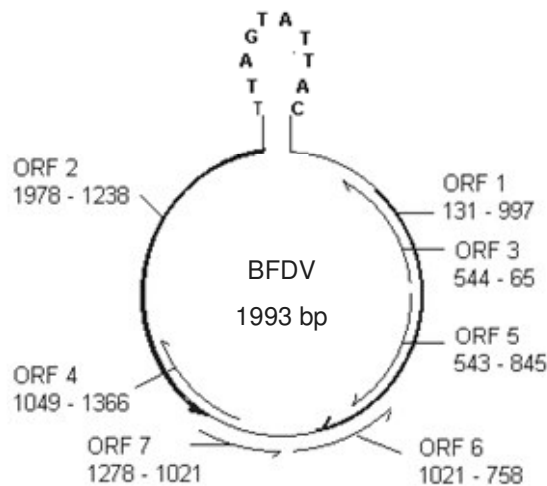
Figure 1.2.2: Genome of *Beak and feather disease virus* indicating seven open reading frames in an ambisense organisation and a conserved nonanucleotide motif (TAGTATTAC). Adapted from Bassami *et al.* (1998).

The Rep protein is involved in rolling circle replication (RCR) of the virus while the CP forms the capsid of the virion. ORF 1 is the most conserved region of the genome while the less conserved ORF 2 is responsible for the genome diversity observed in BFDV isolates.

1.2.1.3.   Genetic Diversity.

The isolation of BFDV from four different psittacine species by Ritchie *et al*. (1990) that shared similarities in ultrastructural characteristics, protein composition and antigenic comparison and were therefore identical has contributed to the present assumption that there is only one strain of BFDV worldwide. However, recent studies on both the Rep and CP genes indicate a higher level of genetic diversity than was initially suggested.

Although species specificity of BFDV needs to be confirmed, as reports contradict each other, separation into different lineages is evident (Bassami *et al*., 2001; Ritchie *et al*., 2003; Heath *et al*., 2004; Raue *et al*., 2004). Ritchie *et al*. (2003) used phylogenetic analysis of the Rep gene to identify a cockatoo, budgerigar and lorikeet lineage while Heath and co-workers (2004) identified 8 BFDV lineages based on phylogenetic analysis of the CP gene and reported on the occurrence of three unique genotypes in southern African isolates whose genetic variations are as a result of point mutations and recombination events. The diversity seen in South African isolates (Heath *et al*., 2004; Kondiah *et al*., 2006) is similar to that present in Australian and New Zealand BFDV isolates [Figure 1.2.3.]. However, no serological tests have been performed to confirm that the genetic differences observed translate into antigenic differences, an important aspect to investigate for the development of a vaccine against PBFD.
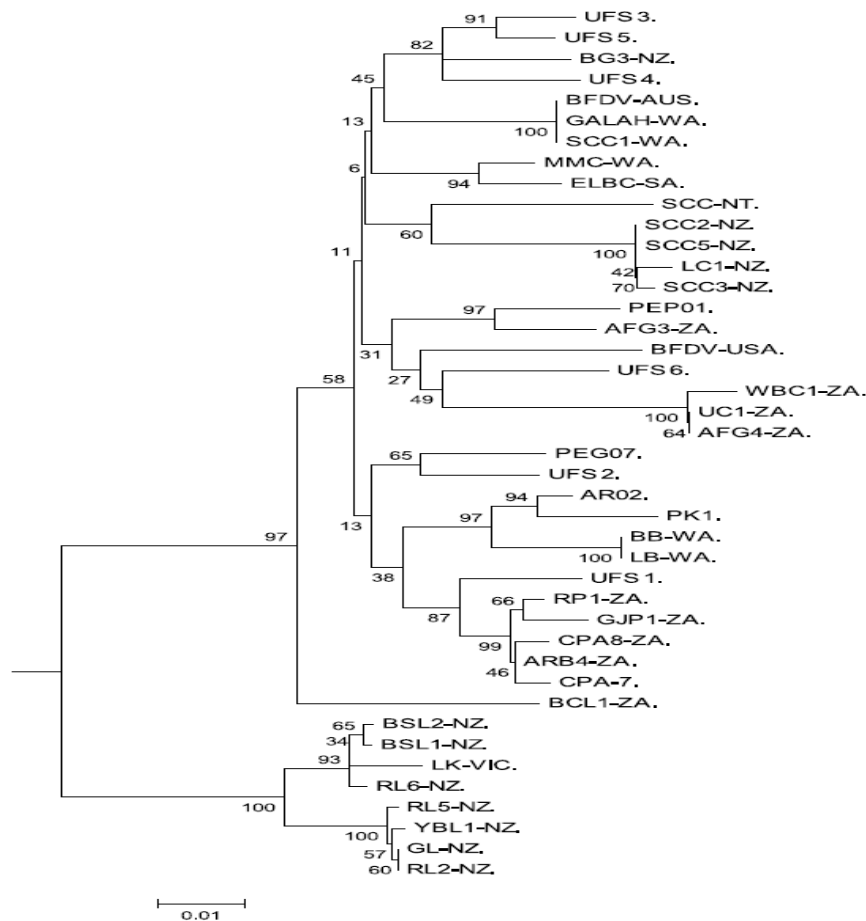
Figure 1.2.3: A neighbour-joining tree depicting the phylogenetic relationship of the Rep sequences of UFS 1–6 with those of known BFDV isolates. The full length tree was rooted with three non-psittacine avian circoviruses (Canary circovirus [GenBank accession number AJ301633], Goose circovirus [GenBank accession number AJ304456] and Columbid circovirus [GenBank accession number AJ298299]). Only the subtree showing the BFDV isolates and their bootstrap values is shown (Kondiah *et al.*, 2006).

1.2.1.4. *Genome Replication.*

Circoviruses are highly dependent on cellular enzymes for replicating their DNA due to their limited coding capacities. The exact mechanism of BFDV replication is unknown but based on the structural and genome sequence similarities it shares with bacteriophages and plant geminiviruses, their replication processes can be used as a replication model.

Synthesis of the C strand generates the first replicative form (RF) DNA and thereafter replication progresses by RCR. The Rep protein nicks and binds a conserved

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nonanucleotide motif (TAGTATTA^C) in the BFDV genome, due to the presence of a tyrosine (Y) residue conserved in all isolates (Ypelaar *et al.*, 1999), allowing RCR to progress. As elongation proceeds, the virus strand is displaced and cleaved from the newly synthesized strand and self-ligates to form circular ss-DNA similar to RCR in bacteriophage φX174 (Bassami *et al.*, 1998, Niagro *et al.*, 1998). These strands can either be encapsidated into virus particles or serve as templates for complementary strand synthesis to generate more RF DNA.

1.2.2. <u>DISEASE ASPECTS.</u>

PBFD has been reported in both wild and captive psittacine birds worldwide (Ritchie *et al.*, 1992). Its host range includes more than 40 species of Old and New World psittacines. Serological evidence indicates that Old World psittacines are more likely to be infected due to their high susceptibility to BFDV while Australian reports indicate widespread infection in wild populations as a result of a seroprevalence of 41-94% BFDV infection in flocks of different free-ranging birds (Ritchie and Carter, 1995).

1.2.2.1.   *Clinical Features.*

PBFD is a fatal dermatological condition that occurs predominantly in birds younger than three years of age. Adult birds are known to be affected but generally remain as carriers of the virus. Affected birds lose contour feathers in a roughly symmetrical pattern and normal plumage is replaced by abnormal feathers [Figure 1.2.4.] (Pass and Perry, 1984).

Beak abnormalities such as progressive elongation, development of fault lines and breakage as well as claw deformities can also occur. Birds may also suffer from anorexia, weight loss and depression.

Figure 1.2.4: Symptoms of PBFD affected birds. Abnormal feathering can be seen in the *Psittacus erithacus* (African grey parrot) [A] and baldness as well as beak abnormalities in the *Eclectus roratus* (Eclectus parrot) [B].

PBFD occurs in a peracute, acute and chronic form which differs markedly in the patterns in which clinical presentation occurs. The type of infection is heavily dependent on the age of the bird at which feather abnormalities first occur but can also be influenced by the route of viral exposure, the titre of the infecting virus and the condition of the bird when viral exposure occurs (Ritchie and Carter, 1995).

The peracute form is common in neonatals showing signs of septicaemia, pneumonia, enteritis and rapid weight loss resulting in death of the bird and no feather abnormalities are observed. The acute form of PBFD is usually seen in young birds during their first feather formation and is characterised by several days of depression, sudden changes in the developing feathers such as necrosis, fractures, bending and haemorrhaging or the shedding of diseased feathers. Birds that survive the acute form of PBFD may develop the chronic form of the disease which is characterised by the symmetric, progressive appearance of abnormally developed feathers during each successive moult (Ritchie and Carter, 1995). These birds become carriers of the virus that can be shed later on and become a source of infection.

1.2.2.2.   Pathology.

A combination of dystrophy and hyperplasia in the epidermis of the feather follicle, beak and claws is responsible for the feather, beak and claw abnormalities seen in PBFD-affected birds (Pass and Perry, 1984). These degenerative changes as well as the immunosuppression of the host due to atrophy of the thymus and bursa of Fabricius can result in bacterial infections.

Histologic evidence suggests that BFDV may be epitheliotropic in feathers and follicles, targeting replicating cells within the basal layer of the epithelium (Latimer *et al.*, 1991). Basophilic intranuclear and intracytoplasmic inclusion bodies have been detected in epithelial cells whereas intracytoplasmic inclusions are often detected in macrophages within the feather pulp cavity or epithelium. Viral inclusions have also been observed in many tissues like the beak, crop, liver, thymus, spleen, intestines, parathyroid gland and bursa of Fabricius [Figure 1.2.5.] (Ramis *et al.*, 1998).
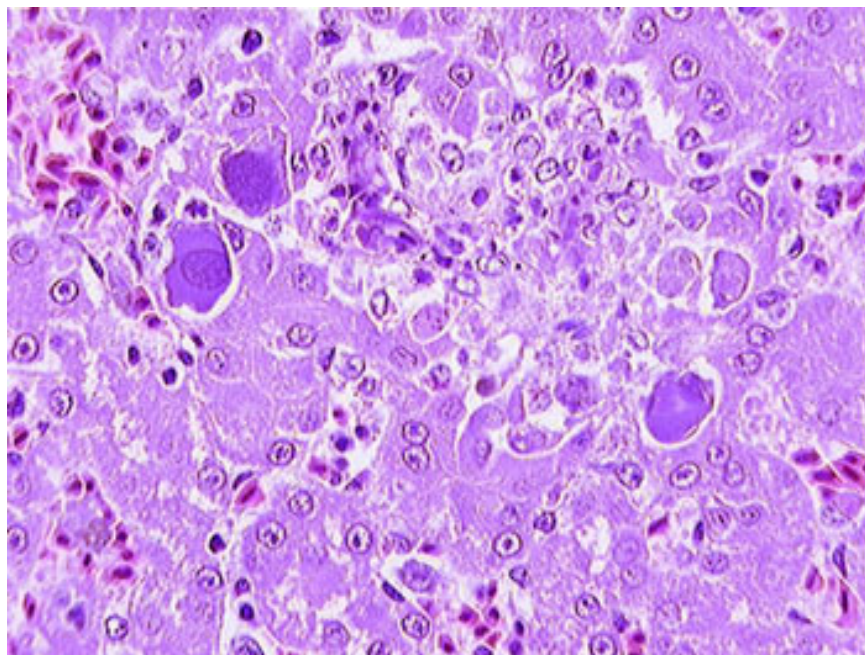
Figure 1.2.5: Beak and feather disease virus inclusion bodies in an H&E stained liver section
of an affected bird at 1000X magnification.
http://www.vet.uga.edu/vpp/ivcvm/1999/gregory/index.php

Circoviruses are known to target pre-cursor T cells leading to immunosuppression in the host as a result of the depletion of both helper T cells and cytotoxic T cells

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(CTLs). The immunocompromised host eventually dies as a result of secondary bacterial, chlamydial, fungal or other viral infections.

1.2.2.3.　　Epidemiology.

BFDV can be transmitted both horizontally and vertically from hen to chick. Several reports indicate that asymptomatically infected birds can produce clinically infected progeny in successive breeding seasons, suggesting the existence of a carrier state from which both horizontal and vertical transmission may occur (Ritchie and Carter, 1995).

Direct contact with infected birds, viral contaminated water or feeding areas leads to horizontal transmission of the virus. This process is accelerated by the flocking nature of many birds and can lead to increased mortalities. Preening and feeding activities also allow the inhalation and ingestion of viral particles leading to infection. Feather dust is indicated to be a major vehicle of transmission and environmental persistence of BFDV due to the high concentration of virus found in it and the ease with which it can be dispersed (Ritchie *et al.*, 1991a).

1.2.2.4.　　Diagnosis.

Routine diagnosis of BFDV infection and the development of diagnostic tests and vaccines are restrained by the inability to propagate the virus in tissue or cell culture or in embryonated eggs as reported by Johne *et al.* (2004). The two most common diagnostic tests for PBFD are the HA and HI assays that detect antigen and antibodies respectively and the polymerase chain reaction (PCR) that detects nucleic acids.

The HA and HI assays are both quantitative and have been used to detect BFDV in faeces and crop washings (Raidal *et al.*, 1993a) and in vaccine challenge studies (Ritchie *et al.*, 1992; Raidal *et al.*, 1993b). The HA assay involves reacting purified viral particles with erythrocytes from a suitable source of parrot species in two-fold dilutions. The HA titre is considered to be the highest dilution that induces agglutination of the erythrocytes. The HI assay involves using a specific number of HA units and sera in two-fold dilutions to detect antibodies against BFDV in the sera which will inhibit agglutination of the erythrocytes.

One drawback of using the HA and HI assays is the differences in the agglutinating ability of erythrocytes obtained from different species and individuals of a species (Sanada and Sanada, 2000). *Cacatua galerita* (sulphur crested cockatoo) erythrocytes are reported to be the most sensitive and the use of erythrocytes from *Psittacus erithacus* (African grey parrot) and *Poicephalus cryptoxanthus* (Brown-headed parrot) has been reported by Kondiah and co-workers (2005). An interesting observation is that the successful use of erythrocytes in HA and HI assays from Australian (Raidal *et al.*, 1993b; Sanada and Sanada, 2000) and African (Kondiah *et al.*, 2005) birds has been reported on but the use of South American birds as a source of erythrocytes proved unsuccessful as reported by Soares *et al.* (1998). Kondiah and co-workers (2005) speculated on a link between the pathogenicity of BFDV in different psittacine species and the application of their erythrocytes in HA and HI assays as African and Australian species of parrots are reported to be more susceptible to BFDV infection than New World birds which are said to be more resistant to viral attack. Another disadvantage of the HA and HI assays is the occurrence of false-positive reactions due to additional cross-reacting antigens and the inability to detect latent BFDV infections.

PCR has been used to detect viral DNA in feather follicle material from clinically affected birds, in blood from asymptomatically infected birds and in swabbed material collected from cages and enclosures to ensure that environments are free from infection (Todd, 2000). The assay is based on the amplification of a conserved region within ORF 1 of the genome. Real-time PCR which was highly sensitive and reliable has also been used to amplify a region from ORF 2 (Raue *et al.*, 2004). A negative PCR test is a strong indication that a bird is not infected but a positive result should be interpreted with the clinical signs, age of the bird and circulating antibody titres.

Fluctuating results have been observed in asymptomatic birds using the PCR test which could be due to the absence of circulating virus in the blood (Kondiah, 2004 – Master's thesis). Viral inclusion bodies have been identified in most organs and tissues of birds and therefore the virus is likely to be present in these tissues as opposed to circulating in the blood where it can be easily removed from the bird's system due to circulating antibodies. It is therefore important to develop a diagnostic test (such as an enzyme linked immunosorbent assay [ELISA]) that would be accurate in detecting any past exposure to PBFD by the detection of specific antibodies whether the bird is showing clinical signs or is asymptomatic. ELISAs are highly specific and quantitative tests but due to the lack of large amounts of infected

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tissues from which to purify viral particles for use in the ELISA, there is no commercially available ELISA for use as a diagnostic test. The use of recombinant DNA technology has enabled the expression of antigenic protein for use in serological tests as reported by Johne *et al.* (2004). The study reported on the use of bacterial expressed recombinant truncated CP as coating antigen in an indirect ELISA that was used to successfully detect BFDV specific antibodies in parrot sera from naturally infected birds. Bound parrot antibodies were detected using anti- IgG of African grey parrots (*Psittacus erithacus*) raised in rabbits followed by addition of conjugated anti-rabbit antibodies. The truncated recombinant CP was also successfully used in HI assays and immunoblotting and the results of the three tests correlated relatively well indicating specific detection of antibodies against BFDV.

1.2.2.5. Treatment and Control.

The inability to propagate BFDV *in vitro* has hindered the development of a killed or attenuated vaccine suitable for commercial application. The lack of a control programme restricts the options available for the prevention of PBFD and so the disease is best treated supportively. Effective biosecurity measures include the repeated cleaning of cages, feeding utensils and other facilities with disinfectants like Virukill avian®. Warm, draught-free environments should be provided for birds as their thermoregulatory systems are impaired due to extensive feather loss. Balanced diets and additional supplements should be administered to birds to help them combat secondary infections as they become more susceptible due to immunosuppression caused by viral attack on the developing bursa.

However, the most practical form of control would be the use of a vaccination programme whose success would be guided by a number of factors: the ability of naturally exposed psittacines to remain clinically normal and develop a protective immune response, the indication that an antigenically similar BFDV infects a wide range of psittacine birds and the temporary protection afforded to chicks from vaccinated hens. Future research therefore lies in the production of DNA and sub-unit vaccines that will not rely on a steady supply of limited wild-type virus but on molecular technology to produce a sufficiently immunogenic antigen for the prevention of PBFD.

1.3. THE DEFENSE MECHANISMS OF A BIRD.

Viruses have four main preferred sites for entering a bird which include skin-to-skin contact, ingestion (faecal/oral), inhalation and the use of mechanical or biological vectors. Of these four routes, *Circoviridae* are known to be transmitted via all except skin-to-skin contact making BFDV a highly transmissible virus. Irrespective of the route by which a virus may enter the bird or the condition of the bird's defence systems, the more virus a bird is exposed to, the more likely the virus is to bypass the bird's defence mechanisms and cause an infection (Ritchie and Carter, 1995).

1.3.1. <u>EXTERNAL DEFENSES.</u>

A bird has both internal and external defence mechanisms. The external mechanisms involve non-specific protective barriers such as skin and mucosa, the normal microbiota found on the skin and mucous membranes and specific cells and their secretions that prevent attachment of foreign bodies to cells.

Healthy feathers and skin are dead and do not support growth and replication of viruses thereby preventing infection by viruses that are not transmitted via skin-to-skin contact. Specialised cells in the skin secrete fatty substances that allow the proliferation of the normal skin microbiota that retard the growth of pathogens (Ritchie and Carter, 1995). The respiratory tract entraps inhaled viruses in mucus material which is expelled by sneezing or swallowing. The mucus lining the trachea in birds also contains virus specific antibodies that bind to and neutralise the specific viruses to which they have been produced.

Saliva and mucus present in the oral cavity bind to and inactivate some ingested viruses while hydrochloric acid (HCl) in the proventriculus of a normal bird rapidly destroys most types of ingested viruses (Ritchie and Carter, 1995). Other ingested viruses can be destroyed by proteolytic or lipolytic compounds in the upper gastrointestinal tract. The normal gastrointestinal microbiota and the continuous regeneration of gastrointestinal cells also help to prevent infection by ingested viruses.

Although a bird has such strong external defences, BFDV can easily penetrate them and cause infection. CAV and PCV-1 are both highly stable viruses as they resist

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heat treatment at 70°C for 15 minutes and treatments at pH 3.0 (Todd, 2000). BFDV is similar in structure to both CAV and PCV-1 and has been reported to be a highly stable virus as well. It can still agglutinate erythrocytes after incubation at 80°C for 30 minutes is resistant to chemical inactivation and disinfectants (Todd, 2000). Circoviruses are likely to be extremely resistant to environmental degradation allowing BFDV to be an easily transmissible virus that can survive in the environment for a longer period until it finds a host. Because of BFDV's high stability and ability to penetrate the external defences, a bird's internal defence system plays a large and important role in combating PBFD.

1.3.2.   INTERNAL DEFENCES.

A bird's internal defence system comprises the immune system which is an interactive network of lymphoid organs, cells, humoral factors and cytokines (Parkin and Cohen, 2001). The immune response can be divided into the innate response which provides immediate host defence and the adaptive response which involves precise antigen-specific reactions.

1.3.2.1.   The Innate Response.

This response is usually rapid but lacks specificity and involves neutrophil recruitment, the complement system, natural killer cells, eosinophils and mast cells and basophils.

Neutrophils are mobile cells that circulate in the blood or along the vascular endothelium. These cells are phagocytic and function by engulfing antigens into a phagolysosome where the antigen is killed or destroyed by toxins and enzymes. The complement system is activated to form transmembrane pores in the antigen's cell surface leading to death by osmotic lysis. Eosinophils are involved in protection from parasitic (nematode) infections while mast cells and basophils are involved in anaphylaxis and angioedema. Natural killer cells lyse antigens by secreting perforins onto the surface of the cell which make holes in the cell membrane. Granzymes that induce apoptosis in the target are then injected into these pores (Parkin and Cohen, 2001) killing the cell.

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The Adaptive Response.

Adaptive immunity is characterised by the use of antigen-specific receptors on T and B cells that recognise the antigen, leading to activated T cells homing to the disease site or the release of antibodies from activated B cells [Figure 1.3.1.].

Antigens derived from pathogens that replicate intracellularly (viruses and tumour cells) are produced endogenously and become complexed with major histocompatibility complex (MHC) class I through intracellular processing pathways (Parkin and Cohen, 2001). Short peptides are released into the cell cytoplasm where they are transported into the endoplasmic reticulum (ER) and are picked up by MHC class I molecules. The antigenic peptides are exhibited on the cell surface in the MHC class I complex where they are recognised by CD8 T cells which become activated, acquiring cytotoxic functions. These CTLs can lyse the infected host cell thereby limiting the replication of the virus and controlling the spread of infection.
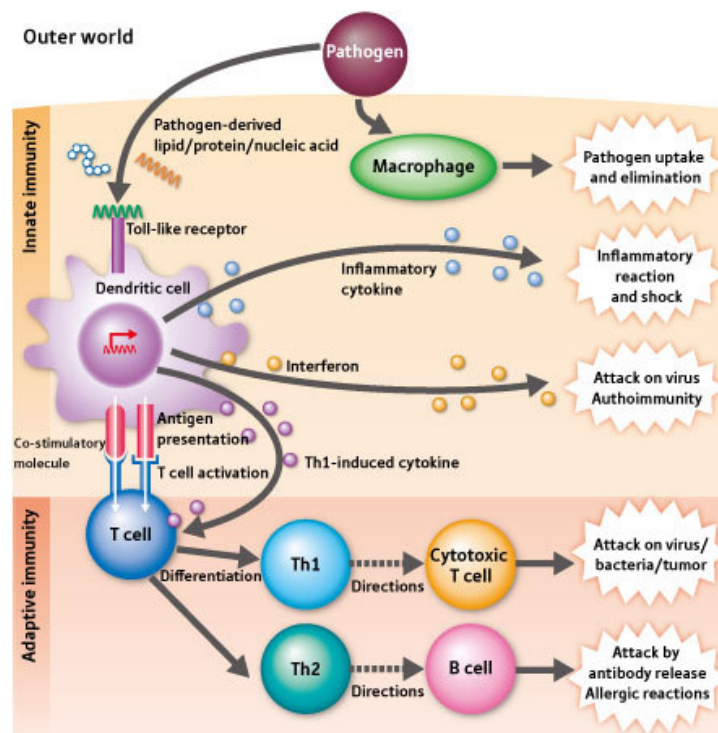
Figure 1.3.1: Cellular and humoral immunity responses.
http://www.rikenresearch.riken.jp/frontline/303/images/fig1.jpg

Exogenous antigens (like virions, bacteria and microbial proteins) are taken up by specialised antigen presenting cells (APCs) such as dendritic cells, B cells and

macrophages by endocytosis. These antigens are degraded into smaller peptides which bind vesicles of MHC class II molecules (formed in the ER) in the cell cytoplasm forming a complex. The antigens are presented on the cell surface by MHC class II molecules where they are recognised by CD4 T helper cells. The T helper cells secrete cytokines that activate the maturation of B cells into plasma cells that secrete antibodies. Antibodies can bind to antigen, neutralizing the infectivity of a pathogen while antigen that binds to membrane-bound antibody on the surface of B cells stimulates B cell division, amplifying the antibody response (Hassett and Whitton, 1996).

All nucleated cells express MHC class I allowing any cell that becomes infected with a virus or other intracellular pathogen, or is producing abnormal tumour antigens to be removed by cytotoxic attack (Parkin and Cohen, 2001). The production of cytokines for the activation of the antibody response needs to be regulated and so only a small number of MHC class II APCs are available to drive the appropriate response. Innocuous antigens are largely ignored due to the need for intracellular processing and expression with MHC for an antigen to be recognised as foreign, another regulatory system of the adaptive response (Parkin and Cohen, 2001).

Circoviridae are known to target lymphoid tissue leading to the depletion of both CD4 and CD8 T cells (Ritchie *et al.*, 2003). The depletion of these cells and the atrophy of the thymus and bursa of Fabricius lead to an immunocompromised host that is susceptible to attack by secondary infections. Commonly, it is these secondary infections that result in death of the bird during BFDV infection.

Severe cryptosporidial infections have been reported in *C. galerita* (sulphur crested cockatoo), which is common in patients with immunodeficiencies (Ritchie and Carter, 1995). PBFD positive birds with intracytoplasmic inclusion bodies located in the macrophages usually succumb to death as the macrophages are critical for the initial processing and presentation of the viral antigen (Ritchie and Carter, 1995). Therefore, the immune system becomes a determining factor in whether a bird develops a chronic fatal BFDV infection or develops a protective immune response. Once an effective immune response has been mounted to eliminate the virus, the birds remain asymptomatic. If a bird does not mount an immune response, death can occur within as little as 3-4 weeks after exposure as reported in experimentally infected 6 week old *C. galerita* (sulphur crested cockatoo) chicks.

The importance of being able to stimulate a quick and efficient immune response in psittacine chicks becomes imperative in providing protection against BFDV infection. This can be facilitated by producing a vaccine that will allow the chicks to produce antibodies against infecting BFDV even after maternal antibodies have been depleted (~ 3 weeks of age).

1.4. VACCINE DEVELOPMENT AGAINST BFDV.

A vaccine is a preparation of material that is injected into an organism to help prevent harm from infectious diseases or products of harmful, infectious organisms by stimulating the immune system. The ideal vaccine might be characterised as safe, cheap, heat-stable, containing protective immunogenic sequences from multiple pathogens and preferably administered as a single dose (Hassett and Whitton, 1996). Although vaccines available today do not meet all these criteria, they have been successful in reducing the occurrence of infectious diseases and can be classified as "live" or "dead".

Dead or killed vaccines consist of killed whole pathogens or soluble pathogen proteins or protein subunits (e.g. *Vibrio cholerae*) while live vaccines consist of attenuated viral or bacterial particles, selected for reduced pathogenicity but maintain their immunogenicity (e.g. smallpox, polio). They also include recombinant vaccines where foreign antigens are expressed from a replicating bacterial or viral vector (hepatitis B surface antigen).

Attempts to develop an inactivated vaccine against BFDV have been made by Ritchie and co-workers (1992) and Raidal and co-workers (1993b). However, the inability to propagate BFDV *in vitro* makes this a very expensive process as there is no constant supply of wild-type virus. Treatment of an inoculum consisting of a feather homogenate prepared from feathers of four species of psittacine naturally affected by BFDV with ß-propiolactone destroyed viral infectivity and failed to produce disease in inoculated *Melopsittacus undulatus* (budgerigars) and *Cacatua roseicapilla* (galahs) as reported by Wylie and Pass (1987). Ritchie and co-workers (1992) treated purified BFDV with ß-propiolactone and inoculated it intramuscularly and subcutaneously into *Cacatua alba* (Umbrella cockatoo) and *Psittacus erithacus* (African grey parrot) in Freund's adjuvant. They reported that the chicks of vaccinated birds were temporarily resistant to BFDV challenge whereas non-vaccinated birds developed clinical disease

suggesting that maternally transmitted immunologic factors can protect neonates from virus challenge.

Raidal *et al.* (1993b) reported their findings on vaccination with ß-propiolactone treated BFDV in a double-oil emulsion (DOE) adjuvant system. Their responses were as effective and protective as the vaccine used by Ritchie *et al.* (1992) but the DOE vaccine was well tolerated by the chicks and caused little inflammation as opposed to the vaccine in Freund's adjuvant which caused undesirable tissue reactions.

Prevention of PBFD by vaccination can be successful because of a number of factors: investigations indicate that many naturally exposed birds remain clinically normal and develop a protective immune response, studies indicate that BFDV isolated from different genera of psittacines appear to be antigenically similar and experimental vaccination studies have been successful in protecting vaccinated birds from disease and report the transfer of protection from vaccinated hens to chicks. The inactivated vaccine was developed in Australia but due to the resilience of the virus and its unique methods of replication, the vaccine is unsuitable for use in the USA and Europe (Ritchie and Carter, 1995).

Apart from the lack of a cell culture system for BFDV to produce high enough amounts of virus for inactivation, the use of inactivated vaccines has some disadvantages. These vaccines can not efficiently enter the MHC class 1 pathway thereby lacking the efficiency in stimulating a cell mediated response critical for protection against viral diseases as viruses are intracellularly replicating organisms (Hassett and Whitton, 1996). Economically, both inactivated and live vaccines are expensive to produce as they require refrigeration and have a limited shelf life.

Therefore, developing a vaccine for the prevention of PBFD should make use of the new approaches being taken toward vaccination. Producing a sub-unit vaccine or a DNA vaccine would be a more economical yet safer and more effective approach towards preventing PBFD. For the purpose of this study, emphasis is on the DNA vaccine. DNA vaccines are composed of antigen-encoding genes whose expression is regulated by a strong mammalian promoter expressed on a plasmid backbone of bacterial DNA (Ichino *et al.*, 1999).

1.4.1. <u>CHARACTERISTICS OF DNA VACCINES.</u>

DNA vaccines usually consist of plasmid vectors that contain heterologous genes inserted under the control of a eukaryotic promoter, allowing protein expression in mammalian cells (Garmory *et al.*, 2003).
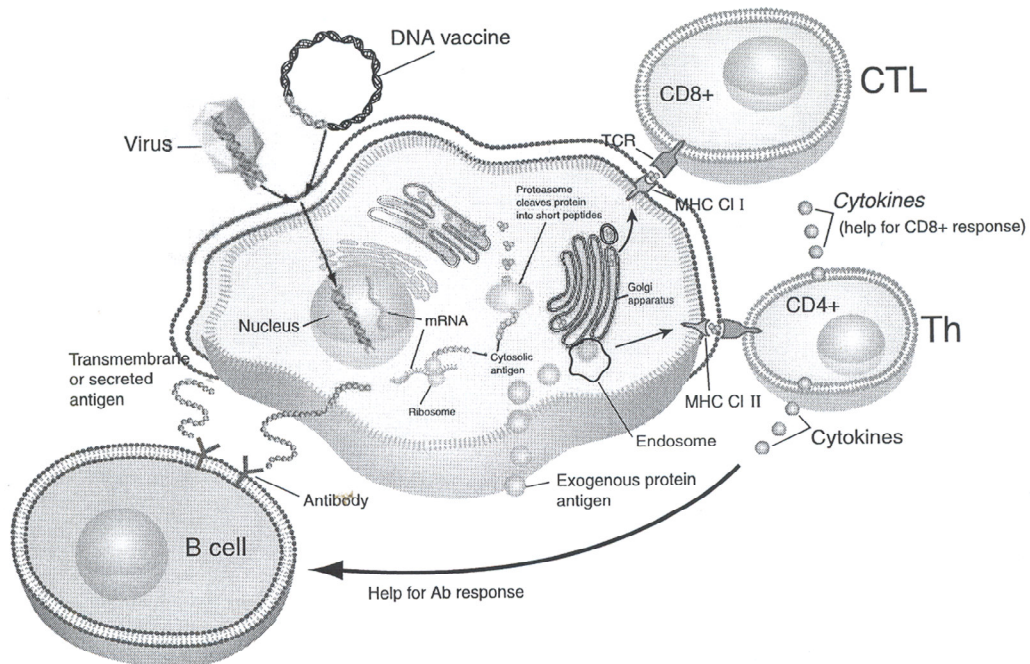
Figure 1.4.1: Mechanism of generation of cytotoxic T-lymphocytes, helper T-cells and antibodies (Liu, 2003).

In vivo synthesis produces proteins with conformations and post-translational modifications similar, in most cases, to those that occur during natural infection. This allows the presentation of foreign antigen to both MHC class I and II molecules stimulating both the cytotoxic and antibody responses [Figure 1.4.1] thereby mimicking natural viral infection. Table 1.2 compares conventional vaccines with some of the new vaccine strategies being developed today.

Table 1.2: Comparison of old and new vaccine strategies. (Adapted from Hassett & Whitton, 1996).

	Vaccine type			
	Whole live attenuated	Live recombinant vector	Killed pathogen	DNA vaccine
Endogenously synthesised antigens resulting in cytotoxicity	Yes	Yes	Usually not	Yes
Ability to access MHC class II inducing Helper T cells	Yes	Yes	Yes	Yes
Antigenic competition between vector and target antigen	No	Possibly	No	No
Safety in pregnant and immunosuppressed individuals	No	No	Probably	Probably
Risk of reverse pathogenicity	Yes	Yes	No	No
Efficacy reduced by maternal antibody	Usually	Possibly	Possibly	No
Easy to prepare and purify	No	No	No	Yes
Heat stability	Usually not	Usually not	Usually	Yes
Cost advantages	No	No	No	Yes

The choice of plasmid vector plays an important role in DNA vaccine design and should have a eukaryotic promoter, a cloning site, a polyadenylation sequence, a selectable marker and a bacterial origin of replication [Figure 1.4.2.] (Garmory *et al.*, 2003).
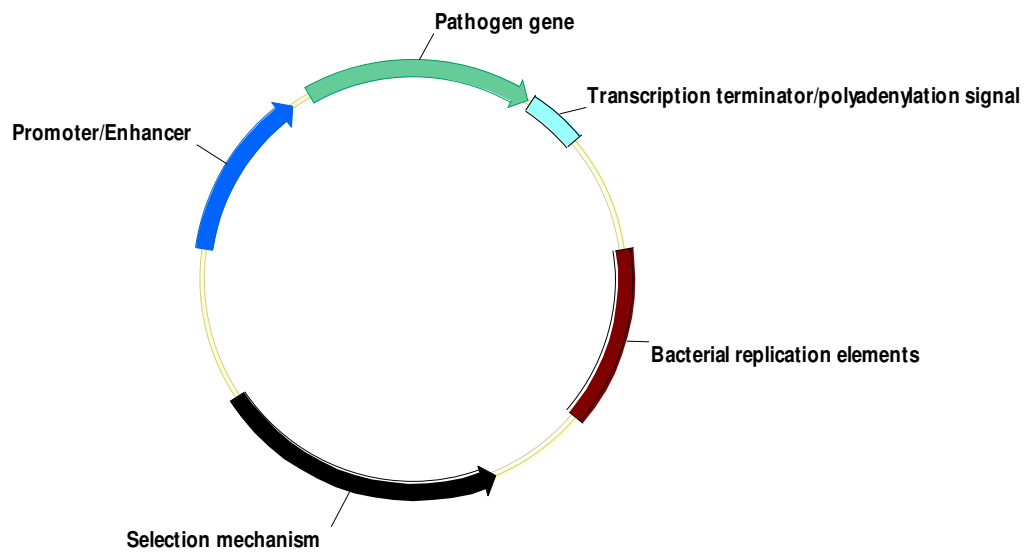
Figure 1.4.2: Map of a typical DNA vaccine construct. The vector includes a promoter such as CMVIE and SV40, a transcription terminator such as BGH, and a selection marker such as antibiotic resistance genes. Adapted from Prather *et al.* (2003).

Strong eukaryotic promoters are commonly used for DNA vaccines to enable high levels of protein expression. Common promoters include the human cytomegalovirus (CMV) promoter, RSV promoter and the beta actin promoter among others. The CMV promoter has often been shown to direct the highest level of transgene expression in eukaryotic tissues when compared to other promoters (Garmory *et al.*, 2003). Macaques injected with a DNA vaccine encoding human immunodeficiency virus type 1 (HIV-1) Gag/Env under the influence of the CMV promoter elicited higher Gag- and Env- specific humoral and T-cell proliferative responses than macaques injected with a similar DNA vaccine which was under the control of the endogenous AKV murine leukaemia long terminal repeat (Garmory *et al.*, 2003). This indicated the greater transcriptional activity of the CMV promoter. However, the CMV promoter is not suitable for some gene therapy applications due to the inhibition of expression from this promoter by interferon-γ and tumour necrosis factor-α. In such situations, the CMV promoter can be replaced by the creatine kinase promoter, desmin promoter/enhancer or metallothionein promoter (Garmory *et al.*, 2003).

A strong promoter increases the rate of transcriptional initiation which may result in the rate of transcriptional termination becoming rate-limiting. A polyadenylation sequence used within the DNA vaccine stabilises the transcription of messenger RNA (mRNA) as well as the processing and polyadenylation of mRNA transcripts. The

selectable markers in the DNA vaccine backbone are usually antibiotic resistance genes and are used to select positive transformants during production in bacteria and during transient transfection of cells. The bacterial origin of replication allows high copy number replication of the plasmid and growth in bacterial cells.

Recognition of the AUG initiator codon by eukaryotic ribosomes can be influenced by sequences surrounding it. The "Kozak" consensus sequence [$^{-6}$ GCCA/GCCAUGG^{+4}] has been defined as the translational initiating sequence with efficient translation occurring when the -3 position is a purine base and a guanine is positioned at +4. Gene expression efficiency often also correlates with the use of selective codons in genes due to the codon bias observed in all species. Microbial DNA contains immunostimulatory motifs that consist of an unmethylated C_pG dinucleotide flanked by two 5' purines [G_pA] and two 3' pyrimidines [T_pC/T_pT]. These motifs are known to trigger the production of Th1-type cytokines of the innate immune response prompting Klinman and co-workers (1999) to examine these properties as adjuvants during DNA vaccination. They demonstrated that physically linking C_pG-containing motifs to the immunogen augmented antigen-specific serum antibody levels by up to ten-fold and interferon gamma production by up to six-fold concluding that the development of novel vaccines and anti-allergens might benefit from the inclusion of C_pG-based adjuvants.

1.4.2. DEMONSTRATIONS OF THE CAPABILITIES OF DNA VACCINES.

The initial demonstrations of the ability of directly transfected DNA encoding immunogenic antigens to induce protective immune responses has led to an increasing interest in the use of DNA vaccines as a potential successful vaccine technology for the future. Numerous DNA vaccines encoding viral proteins have been studied including the haemagglutinin matrix and nucleoprotein (NP) of the influenza virus (Fynan *et al.*, 1993; Kodihalli *et al.*, 1997; Fomsgaard *et al.*, 2000), the gp120 and gp160 from HIV (Donnelly *et al.*, 1997; van Harmelen *et al.*, 2003) and the replication and capsid proteins of PCV-2 (Blanchard *et al.*, 2003; Kamstrup *et al.*, 2004) among others.

DNA vaccines have also been used to raise immune responses against antigens of non-viral pathogens such as *Mycobacterium tuberculosis*, *Salmonella* Typhi (Donnelly

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et al., 1997) and against the circumsporozoite protein of *Plasmodium* species; although Ichino *et al.* (1999) demonstrated the development of tolerance rather than immunity when neonates were administered an anti-malarial vaccine. Cell mediated responses, humoral responses or both have been observed in preclinical studies using various animal models. The type of response may be influenced by the route of DNA administration (Fynan *et al.*, 1993; Gregoriadis *et al.*, 1997; Fomsgaard *et al.*, 2000) and the use of adjuvants (Klinman *et al.*, 1999; Scheerlinck, 2001).

1.4.2.1. The PCV-2 Vaccine Candidate.

Post weaning multisystemic wasting syndrome (PMWS) is a disease that affects piglets aged between 5-12 weeks and was first observed in Brittany in 1996 (Blanchard *et al.*, 2003). Although it first emerged in western Canada in 1991, PMWS has spread to pigs through out the world. The disease is characterised by weight loss, jaundice, dyspnoea and respiratory and digestive disorders. PCV-2 is considered to be the etiological agent in PMWS and belongs to the genus *Circovirus* like BFDV.

Blanchard and co-workers (2003) compared two vaccine strategies in controlling PMWS: a DNA vaccine and a sub-unit vaccine. Their DNA vaccine consisted of plasmids encoding the ORF 1 gene, ORF 2 gene of PCV-2 and the GM-CSF gene as an adjuvant. The sub-unit vaccine consisted of baculovirus expressed crude lysate in a water-in-oil adjuvant. Intramuscular (IM) injection of either of these vaccines induced protection of piglets but when compared to each other, the sub-unit vaccine appeared to provide better protection than the DNA vaccine.

Kamstrup *et al.* (2004) developed a DNA vaccine candidate encoding the capsid protein (ORF 2 gene) of PCV-2 in the vector pcDNA3.1/V5-His/TOPO. Coat protein was expressed *in vitro* in PK15 cells and induced antibody production *in vivo* in mice that were inoculated with the plasmid using a gene gun. They concluded that DNA vaccination would be an attractive method for vaccination against PCV-2 infection, ultimately preventing the development of PMWS.

Like PCV, BFDV has an ambisense genome and the ORF 2 like that of PCV-2 encodes the capsid protein (the epitopic protein) of the virus. When sequence alignments of the two CPs were performed, the CP of BFDV was found to share 26% identity to that of PCV-2 (Crowther *et al.*, 2003). Alignment of the amino acid

sequences also reveals a highly conserved sequence of 14 amino acids (YVtkLTIYVQFRqF) near the carboxyl terminus of both proteins as well as adjacent corresponding myristylation sites suggesting its necessity for protein function (Niagro *et al.*, 1998). ORF 1 in both PCV-2 and BFDV encode the Rep protein, both of which possess the 3 sequence motifs and P-loop motif required for RCR. These similarities between PCV-2 and BFDV suggest that a DNA vaccine based on the model of Blanchard *et al.* (2003) or Kamstrup *et al.* (2004) could find use in protecting psittacines from PBFD.

1.4.3.   MODES OF DNA DELIVERY.

Immune responses have been induced by injecting "naked" DNA in saline, by DNA complexed with lipids and by impelling DNA, either as an aerosol or coated onto gold beads, directly into the epidermis and dermis through the use of an electrical charge or pressurized gas (Hassett and Whitton, 1996).

IM inoculation of naked DNA into skeletal muscle is the most widely used method of immunisation. This is administered directly as plasmid DNA in saline solution or after injection of a toxin or local anaesthetic intended to increase the number of muscle cells expressing antigen and enhance the immunological response. Although there is a lack of data supporting any one theory, it is thought that the enhanced immune response may be as a direct result of increased antigen expression in regenerating muscle cells or via the uptake and expression of DNA by immune cells recruited to the site of tissue damage. Potential disadvantages of naked DNA vaccination include uptake of DNA by only a minor fraction of muscle cells, exposure of DNA to nucleases in the interstitial fluid, the use of relatively larger quantities of DNA and often, the need to inject into regenerating muscle in order to enhance immunity (Gregoriadis *et al.*, 1997).

Hassett and Whitton (1996) reported that when compared with naked DNA, DNA-lipid complexes exhibit reduced expression when injected intramuscularly but enhanced expression and immunity when delivered intravenously. Intravenous (IV) delivery facilitates gene expression in many organs, including the spleen (an important organ of the immune system). In a study by Gregoriadis and co-workers (1997) improved humoral and cell mediated immunity was demonstrated using liposome-entrapped DNA when inoculated intramuscularly into mice. Although there is no evidence of

significant vesicle uptake by muscle cells after local injection with liposome-entrapped DNA, liposomes are known to enter the lymphatic system and localise in the lymph nodes where they may be taken up by APCs. The complexing of DNA enhances its uptake by APCs as well as protecting the DNA from degradation.

Intradermal (ID) inoculation appears to be a favourable route as the skin is easily accessible and the immune system is well represented. It can also induce long-term protection and a strong antigen-specific immune response requiring much less DNA than IM injection. Epidermal cells are also rapidly replaced leading to expulsion of DNA, and alleviating many of the concerns regarding safety of prolonged expression (Hassett and Whitton, 1996).

Approaches towards increasing the potency of DNA vaccines includes using devices that increase the transfection of cells or target the DNA to specific sites [Figure 1.4.3]. A mucosal jet injector device has been utilised that targets the mucosa, a site where most pathogens enter the body allowing for better mucosal responses [as opposed to only systemic responses] (Liu, 2003).
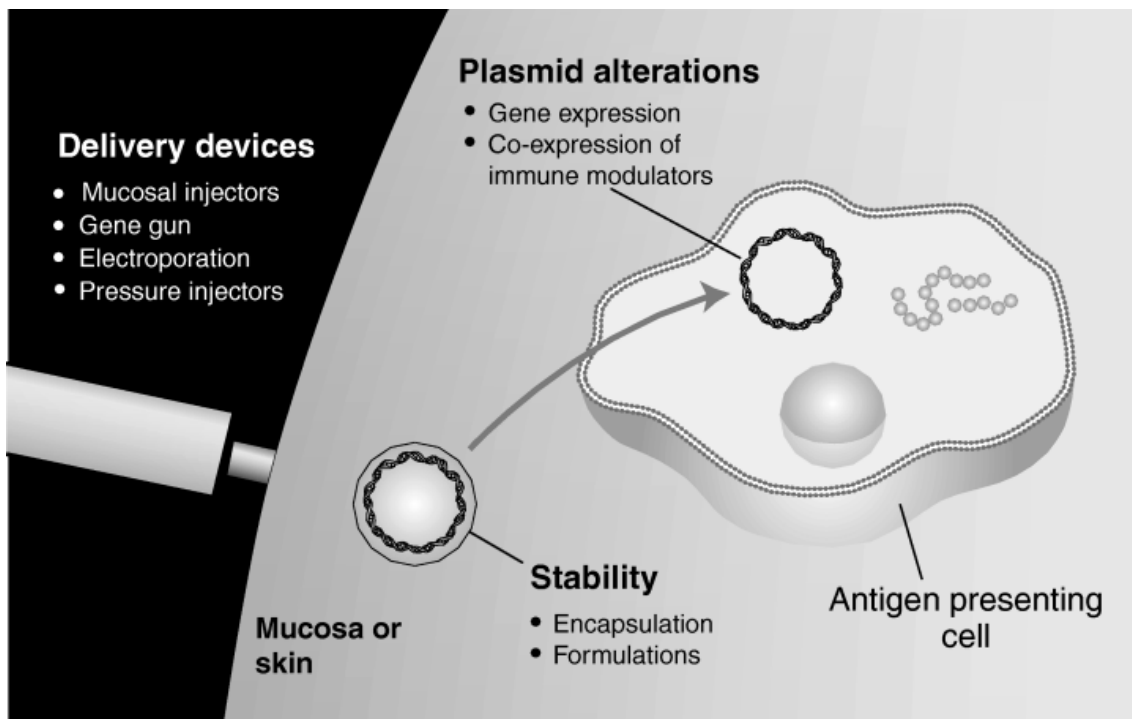
Figure 1.4.3: The improved potency of second generation DNA vaccines includes the use of needle-free delivery devices, electroporation and encapsulation of DNA into microparticles among others (Liu, 2003).

Gene gun delivery of DNA uses an Acell® instrument (Agracelus, Middleton, WI, U.S.A.) to propel DNA-coated gold beads into the skin cells where the DNA is released and expressed as protein (Fynan *et al.*, 1995). It has been found to be a highly efficient means of DNA immunisation using as little as 0.4 µg of DNA. Electroporation devices deliver small amounts of electric current *in vivo* briefly causing the formation of a hole in the cells permitting more of the injected DNA to enter the cells (Liu, 2003).

Delivery of DNA vaccines is moving towards the trend of non-invasive immunisation, a method that would be preferred for animals and humans likewise. Although there are no studies on the potential of different routes of DNA vaccination in birds, IM inoculation appears to produce sufficient stimulation of both the cell mediated and humoral responses with little or no adverse reactions when inoculating animals. This makes the use of DNA vaccination an attractive option as opposed to the use of the inactivated vaccine against PBFD that did not stimulate a cell mediated response and caused adverse tissue reactions when administered to the birds.

1.4.4. <u>POTENTIAL SAFETY CONCERNS AND THE REGULATION OF DNA VACCINES.</u>

Conventional attenuated or live vaccines are known to produce adverse side-effects and although DNA vaccines are an attractive alternative, questions concerning their safety have arisen. These safety concerns include: the development of autoimmune diseases as a result of long-term antigen expression *in vivo* which could lead to the production of autoantibodies or local inflammatory responses of the cells expressing the vaccine-encoded antigen, the development of tolerance other than immunity leading to increased risk of infection and the potential for integration of the plasmid DNA into the host's genome (Smith and Klinman, 2001).

1.4.4.1. Autoimmunity.

Preclinical studies of DNA vaccination of normal mice and mice predisposed to develop systemic autoimmune disease demonstrated that although autoantibodies were secreted, the levels were insufficient to cause disease in normal animals or to increase disease severity in autoimmune-prone mice. Absence of an immune

response towards cells expressing vaccine-encoded antigen suggests that these cells are not targeted for elimination. These results do not eliminate the possibility that DNA vaccines may worsen organ-specific autoimmunity by encoding antigens that cross-react with self (Smith and Klinman, 2001).

1.4.4.2. C_pG Effect.

C_pG containing immunostimulatory motifs have been shown to have adjuvant properties when linked to the vaccine-encoded antigen stimulating a Th1 type immune response. These motifs are common in bacterial DNA but rarely in mammalian DNA due to a combination of C_pG suppression and C_pG methylation. C_pG motifs directly activate macrophages, natural killer cells and lymphocytes raising the possibility that DNA vaccination might bias the host's cytokine profile, thereby contributing to the development of Th1 mediated organ-specific autoimmune disorders, interfering with immune homeostasis or increasing the vaccinee's susceptibility to infections that require vigorous Th2 responses (Smith and Klinman, 2001).

1.4.4.3. Tolerance.

Studies on the development of tolerance in neonatals have produced conflicting results. Some studies indicate the development of immunity in neonatals injected with DNA vaccines (Wang *et al.*, 1997; Zhang *et al.*, 2002) while Ichino and co-workers (1999) demonstrated the development of induced tolerance to a plasmid DNA vaccine encoding the circumsporozoite protein of malaria when administered to new-born BALB/c mice. Tolerance has not been observed following vaccination of immunologically mature animals. Research suggests that the capacity of a DNA vaccine to induce tolerance may be dependent on the nature of the encoded antigen and the age and frequency with which the vaccine is administered (Smith and Klinman, 2001).

1.4.4.4. Plasmid Integration.

DNA vaccination is not intended for genetic integration of plasmid DNA into host chromosomes and present information suggests that the risk of integration is low, although the possibility of it occurring can not be completely excluded (Hassett and

Whitton, 1996). The risk of integration is low due to the construction of these vaccines which are designed to permit localised, short-term expression of the antigen and therefore do not include known integration sequences, long stretches of homology with the host genes or a eukaryotic origin of replication (Donnelly *et al.*, 2003). This means that the chance of homologous recombination into the host chromosomes is unlikely due to the lack of extensive homology between the host genome and the plasmid vector. The efforts being made to increase the potency of DNA vaccines by modifying the vector, co-injecting it with agents that increase cellular uptake or altering the site and/or method of delivery, may however, increase the potential for integration (Smith and Klinman, 2001).

The potential for the use of DNA vaccines is still huge although the above-mentioned risks are important to consider. As there is currently no DNA vaccine available for the prevention of PBFD and no studies have been reported on the development of such a vaccine and any adverse effects it may have when administered to birds, it is important to bear these risks in mind. These safety concerns should be investigated thoroughly when developing such a vaccine and reported to the scientific community. Any important findings will then play a prominent role in the registration of the DNA vaccine against PBFD.

Manufacturers and sponsors of DNA vaccines are faced with a series of novel challenges while health authorities also face the same challenges. The best a sponsor can do is ask theoretically, what could possibly go wrong, then conduct the experiments in appropriate *in vitro* systems and animal models to show that this will not and can not happen (Donnelly *et al.*, 2003).

1.5.   INTRODUCTION TO THE STUDY.

Like Australia where PBFD is known to commonly affect both wild and captive psittacines, the number of incidences of outbreaks of PBFD in South Africa has recently increased causing alarm for both bird breeders and nature conservationists. Bird breeders lose at least 10% of their breeding stocks annually to PBFD and the infection rates will not decline rapidly in the near future unless action is taken to combat the disease.

Nature conservationists in South Africa are calling out for help using the media as a channel for educating bird breeders on the severity of the disease and the need to implement more effective biosecurity measures. The SABC 2, a television channel in South Africa, did an insert (1st January, 2000) on the indigenous Cape Parrot (*Poicephalus robustus*) and the threats it is currently facing as an endangered species. One of the most important threats is PBFD which apart from affecting captive-bred birds is also affecting wild populations in the Eastern Cape (a province in South Africa). The origin of the disease is unknown although there are speculations that the disease could have been introduced into the country via imported birds as South Africa partakes in international bird trade. The disease is a stumbling block to the release of confiscated Cape Parrots (*Poicephalus robustus*) as exposed birds become vectors of the disease capable of infecting wild populations if released back into the wild (BirdLife South Africa, 06 July 2005. Press Release: The Cape Parrot – A desperate plight for conservation intervention).

Today the Cape Parrot Working Group promotes the conservation of the Cape Parrot (*Poicephalus robustus*), raising awareness and lobbying for better legislature where both national and international bird trade is concerned. The Cape Parrot Project of BirdLife South Africa also aims at conserving this threatened species but is this adequate or should scientists in South Africa be contributing towards finding a solution to this existing problem?

Studies by Kondiah and co-workers (2006) indicate the widespread occurrence of PBFD in South Africa where positive birds were identified in 6 provinces in the country. Researchers at the University of Cape Town (UCT) are studying the epidemiology of the disease as well as developing a recombinant vaccine to prevent outbreaks of BFDV infection and have reported on genetic diversity occurring among the various isolates identified by Heath and co-workers (2004) that has been found to be similar to that in Australia.

The present study is being done to develop a DNA vaccine which will be used to achieve protection in neonates. The primary aim of this study is to develop a DNA vaccine candidate that will be capable of stimulating immune responses in vaccinated individuals. Secondary aims include using recombinant technology to express the CP in both bacterial and eukaryotic hosts for use in the development of serological tests such as the ELISA as well as using the ELISAs to detect antibody responses in parrots vaccinated with the DNA vaccine candidate.

1.6. CONCLUSIONS.

PBFD is a severe dermatological condition that affects a wide range of psittacine birds worldwide. The etiological agent is BFDV, a virus that belongs to the family *Circoviridae* that is known to target lymphoid tissue leading to immunosuppression in the host. The immune system plays an important role in protecting a bird against infection and disease, the major role players of the adaptive immune response being the T and B lymphocytes responsible for cell mediated and humoral responses, respectively. Once immunocompromised, a bird becomes susceptible to secondary infections that can be bacterial, fungal or chlamydial in nature, eventually dying as a result of these infections.

Successful disease prevention begins with good biosecurity programmes involving safe yet effective disinfection and vaccination. BFDV is as yet unculturable, a factor that has prevented the development of a commercially available vaccine for the protection of psittacines against PBFD. The discovery of DNA vaccination has offered a promising approach towards disease prevention especially where there is a lack of a ready supply of virus to make conventional vaccines, such as BFDV.

DNA vaccines are cheap, stable, safe to use and have the potential to stimulate both cell mediated and antibody responses, increasing the immunogenicity derived from plasmid DNA. Although there are several aspects being investigated to increase the potency of currently available DNA vaccines, studies have demonstrated their potential to prevent PMWS among various other diseases. Thus, lays the possibility of developing a DNA vaccine candidate that will successfully and effectively prevent BFDV infection.

CHAPTER 2: RECOMBINANT EXPRESSION SYSTEMS FOR THE COAT PROTEIN OF *Beak and feather disease virus.*

2.1. INTRODUCTION.

PBFD can be diagnosed by the HA and HI assays that detect antibodies against the virus using faecal, feather and cloacal samples (Ritchie *et al*., 1991b; Raidal *et al*., 1993a) or by PCR that detects BFDV nucleic acid. However, the HA and HI assays utilise *Eolophus roseicapillus* (galah) erythrocytes that are very expensive in South Africa and Sanada and Sanada (2000) indicated that not all cockatoo species or individuals within a species would be a suitable source of erythrocytes for the HA and HI assays. Kondiah and co-workers (2005) investigated the use of erythrocytes from *Psittacus erithacus* (African grey parrots) and *Poicephalus cryptoxanthus* (Brown-headed parrots) in the HA and HI assays and reported positive haemagglutinating activity from both these sources with purified BFDV particles. It was also reported that no difference in HA and HI activity was observed when the erythrocytes were used from individual or pooled samples from African grey parrots. The PCR test on the other hand, although sensitive is not a quantitative test and fluctuating results have been observed for asymptomatic parrots.

These drawbacks result in the need to develop a diagnostic test that can be both quantitative and sensitive regardless of whether a bird is showing clinical signs of disease or is asymptomatic. The ELISA could be such a diagnostic test that would be able to detect antibodies in birds that have been exposed to natural infection as well as birds that have been vaccinated allowing breeders to accurately monitor the epidemiology of their birds. Currently, there is no commercially available ELISA as the antigen or virus required to coat the plates is lacking in ready supply. Johne and co-workers (2004) reported that attempts to find a tissue/cell culture system to propagate BFDV *in vitro* have been unsuccessful and that the virus can not be propagated in embryonated eggs either.

Molecular technology poses an answer to the lack of a suitable antigen as bacterial, eukaryotic and mammalian systems are now available for the recombinant

expression of antigenic proteins. Open reading frame 2 of BFDV encodes the CP, a 26.3 kDa protein considered to be the epitopic protein that shows 26% nucleotide identity to the CP of PCV-2. Crowther and co-workers (2003) reported that both PCV-2 and BFDV have an icosahedral T=1 structure containing 60 CP molecules arranged in 12 pentamer clustered units forming the capsid of the virus. Bacterial and insect expression systems have been used to express recombinant BFDV CP. Johne *et al.* (2004) recombinantly expressed a truncated form of the BFDV CP in *Escherichia coli* using the pQE-60 vector and used it in an indirect ELISA to detect BFDV-specific antibodies in parrot sera. Their ELISA results correlated relatively well with HI assay results of the same parrot sera, indicating that the ELISA specifically detected antibodies against BFDV. Heath and co-workers (2006) used truncated recombinant CPs to gain insight into the role of this protein in the lifecycle of circoviruses and reported on evidence that suggests that CP directly interacts with the Rep enabling the cotranslocation of the latter into the nucleus.

Bacterial systems for heterologous protein production are attractive because of their ability to grow rapidly and at high density on inexpensive substrates, their often well-characterised genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (Terpe, 2006). Choosing the right expression system is dependent on many factors which include cell growth characteristics, expression levels, intracellular and extracellular expression and posttranslational modifications (Jana and Deb, 2005). The biological activity of the protein of interest and the regulatory issues in the production of therapeutic proteins also play a key role. The Gram-negative bacterium *E. coli* remains the most versatile host for the production of heterologous proteins although some of its major drawbacks include the inability to perform many of the post-translational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium and the limited ability to facilitate extensive disulfide bond formation (Jana and Deb, 2005).

pBAD vectors represent a simple and useful expression system for efficient repression, modulation and moderately high expression, which should permit more careful experimentation on biological processes in bacteria (Guzman *et al.*, 1995). In the presence of arabinose, transcription from the P_{BAD} promoter is turned on while in its absence transcription occurs at very low levels. While maximum levels of expression are high enough to permit studies, they are not as high as those obtained using strong inducible promoters thereby alleviating detrimental effects such as

lethality and formation of inclusion bodies. The vector pBAD/His B was used in this study [Figure 2.1.1].
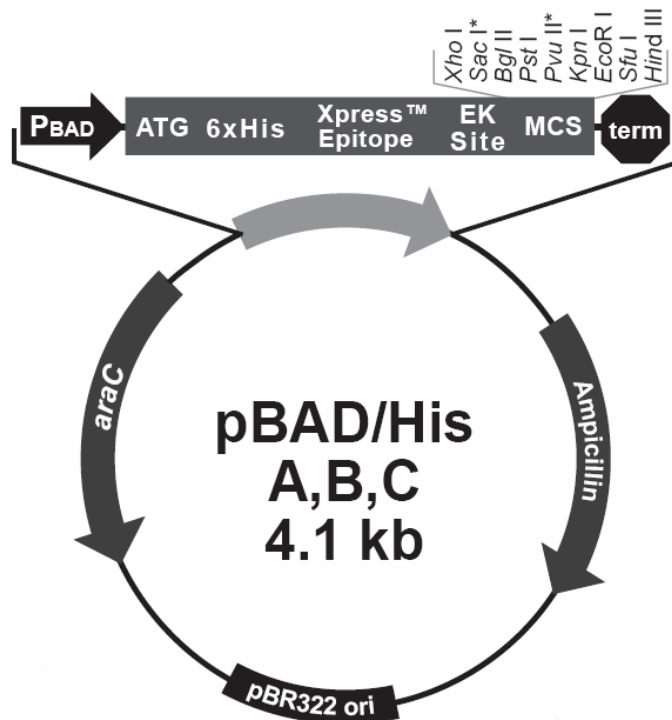
Figure 2.1.1: Schematic map showing the elements of the pBAD/His vector. www.invitrogen.com

Many bacterial systems are not able to modify proteins post-translationally by such means as glycosylation. If post-translational modification is essential for bioactivity, alternative hosts such as yeast, filamentous fungi, or insect and mammalian cell cultures should be used (Terpe, 2006). Yeast expression systems combine the ease of manipulation and growth of unicellular organisms with eukaryotic post-translational processing and modifications (Nicaud *et al.*, 2002). Historically, *Saccharomyces cerevisiae* was used as a host for heterologous protein production, a choice based on the large amount of knowledge accumulated on this organism (Madzak *et al.*, 2004). However, low product yield, poor plasmid stability, hyperglycosylation and low secretion capacities led to the search for a better host. During the last two decades *Pichia pastoris* developed into a highly successful expression system but it also suffers (in some cases) from low secretion levels and hyperglycosylation.

Yarrowia lipolytica is a hemiascomycetous yeast that is heterothallic and dimorphic (forms yeast cells or hyphae and pseudohyphae depending on growth conditions). It is non-pathogenic and has GRAS (generally regarded as safe) status making it an

attractive host for heterologous protein production. *Y. lipolytica* can use glucose (but not sucrose), alcohols, acetate and hydrophobic substances (such as alkanes, fatty acids and oils) as carbon sources (Madzak *et al.*, 2004) and is distinguished for its capacity to secrete naturally several proteins into culture medium. A number of strains (Po1d, Po1e, Po1f, Po1g and YLP21) were derived from the wild-type strain W29 but for the purpose of this study Po1g was used. It was obtained by transforming the Po1f strain with a pBR322-*URA3* vector forming a docking platform to direct integration of the pBR322-based expression/secretion vectors (Nicaud *et al.*, 2002).
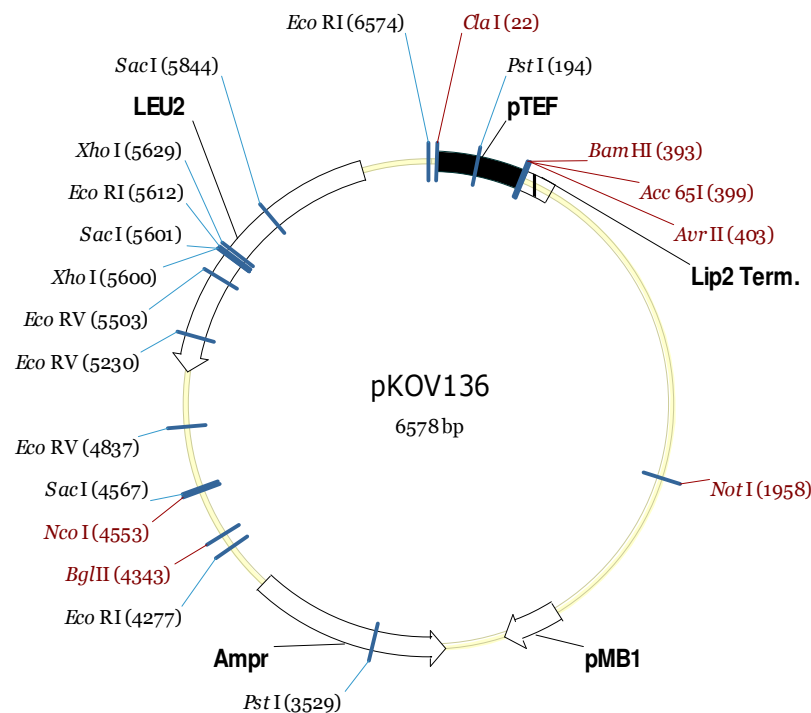
Figure 2.1.2: Schematic map showing the elements of the pKOV136 vector (Adapted from Labuschagne, 2005).

The vector pKOV136 is such a vector [Figure 2.1.2] and allows integration of exogenous DNA into the *Y. lipolytica* genome by homologous recombination. It is greatly stimulated by the linearization of the plasmid within the homology region, which results in very high transformation efficiencies. In more than 80% of the cases, a single complete copy of the vector will be integrated at the chosen site (Madzak *et al.*, 2004; Labuschagne, 2005). Expression of the protein is then under the control of the p*TEF* promoter which is constitutive.

For the purpose of this study two expression systems, one bacterial and one yeast, were used to express recombinant BFDV CP for use in an ELISA. The ELISA will be

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used to detect parrot raised antibodies against infecting BFDV as well as monitor immune responses in parrots to the DNA vaccine.

2.2. MATERIALS AND METHODS.

2.2.1. AMPLIFICATION OF COAT PROTEIN GENE BY POLYMERASE CHAIN REACTION.

PCR amplification of the entire CP from a BFDV isolate which was identified from a ring-necked parakeet in Bloemfontein using PCR amplification of the Rep gene was performed. A forward primer was designed to include a *Bgl*II recognition site and the reverse primer was designed to include a *Hind*III recognition site (Table 2.1) to allow ligation of the PCR product into the vector pBAD/His B for bacterial expression. PCR was carried out in a total volume of 50 µl consisting of 5 µl of DNA template, 1 µl of 10 mM dNTP mix, 0.5 µl of each 100 mM primer, 5 µl of 10X ThermoPol Reaction Buffer and 3.75 U of *Taq* DNA Polymerase (New England Biolabs Inc.) made up to 50 µl with sterile Milli-Q water. The negative control did not contain any DNA template.

Table 2.1: Primer information for bacterial and eukaryotic expression systems.

Primer	Restriction site	Expression system	Sequence[1]
ECEXP07 F1	*Bgl*II	Bacterial[2]	5'- CAG ATC TTG GGG CAC CTC TAA -3'
ECEXP07 R1	*Hind*III	Bacterial	5'- CAA GCT TTT AAG AAG TAC TGG G -3'
YLEXP06 F1	*Acc*651	Eukaryotic[3]	5'- GGT ACC ATG TGG GGC ACC TCT AAC TGC G -3'
YLEXP06 F2	*Bgl*II	Eukaryotic	5'- AGA TCT ATG TGG GGC ACC TCT AAC TGC GCA T -3'
YLEXP07 F3	*Bgl*II	Eukaryotic	5'- CAG ATC TAT GTG GGG CAC CTC TAA CTG CGC -3'
YLEXP06 R1	*Avr*II	Eukaryotic	5'- CCT AGG TTA ATG ATG ATG ATG ATG ATG AGT GCT GGG ATT GTT AGG GGC AAA -3'

Reactions were thermocycled on a Mastercycler Personal (Eppendorf®) starting with an initial denaturation step at 94°C for 5 minutes (mins). Thirty cycles of denaturation

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[1] The restriction site is indicated in red font.
[2] Bacterial expression vector is pBAD/His B.
[3] Eukaryotic expression vector is pKOV136.

at 94°C for 30 seconds (s), annealing at 53°C for 30 s and elongation at 72°C for 90 s were carried out followed by a final elongation step at 72°C for 5 mins. Amplified fragments were observed under ultraviolet (UV) illumination on a 1% agarose gel stained with ethidium bromide. Positively amplified products were confirmed by restriction analysis using the restriction endonucleases *Bam*HI and *Eco*RI and fragments were analysed under UV illumination on a 1% agarose gel stained with ethidium bromide.

Primers were also designed to amplify the entire CP gene from BFDV for expression in a eukaryotic yeast vector pKOV136 (Table 2.1) with the reverse primer designed to include a stop codon and a polyhistidine (6XHis) tag. Each reaction consisted of 5 µl of DNA template, 1 µl of 10 mM dNTP mix, 0.5 µl of each 100 mM primer, 5 µl of 10X ThermoPol Reaction Buffer and 3.75 U of *Taq* DNA Polymerase (New England Biolabs Inc.) made up to a total volume of 50 µl with sterile Milli-Q water. In a number of reactions using primer sets that amplified a large portion of the BFDV genome which included the CP gene, 10 µl of 5X Expand HiFi^{PLUS} Reaction buffer and 2.5 U of Expand HiFi^{PLUS} Enzyme Blend [Expand High Fidelity^{PLUS} PCR System (Roche)] were used. The negative control did not contain any DNA template.

Reactions were thermocycled on a Mastercycler Personal (Eppendorf$^®$) starting with an initial denaturation step at 94°C for 5 mins. Thirty cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 90 s were carried out followed by a final elongation step at 72°C for 5 mins. The reaction cycle when using the Expand High Fidelity^{PLUS} PCR System (Roche) began with an initial denaturation at 94°C for 2 mins followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30s and elongation at 72°C for 2 mins with the final elongation step at 72°C being carried out for 7 mins. A final cooling step at 4°C was also added to the cycle. Amplified fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide. Positively amplified products were confirmed by restriction analysis using the restriction endonucleases *Bam*HI, *Pst*I, *Dpn*I and *Eco*RI and fragments were analysed under UV illumination on a 1% agarose gel stained with ethidium bromide.

2.2.2. <u>PURIFICATION OF DNA.</u>

DNA was either purified from solution or from agarose gels using the Illustria™ DNA and Gel Band Purification Kit (GE Healthcare) following the manufacturer's instructions. A clean scalpel was used to cut the DNA band out of the gel, the agarose slice was placed in a microcentrifuge tube and 300 µl of capture buffer added. The contents were mixed by vortexing vigorously and incubated at 60°C until the agarose completely dissolved. After the agarose had completely dissolved, the tube was centrifuged briefly (Eppendorf 5415D) to collect the sample at the bottom of the tube. A GFX column was placed in a collection tube per purification to be done and the sample transferred to the column. It was incubated at room temperature (rt) for 1 min and centrifuged at full speed for 30 s. The flow-through was discarded, the GFX column placed back in the collection tube and 500 µl of wash buffer added to the column. The column was centrifuged at full speed for 30 s and the GFX column transferred to a clean microcentrifuge tube. Fifty microlitres of elution buffer (10 mM Tris, pH 8.0) was added directly to the top of the column's glass fibre matrix, the sample incubated at rt for 1 min and purified DNA recovered by centrifuging at full speed for 1 min.

Alternatively, a GFX column was placed in a collection tube per purification to be performed from solution and 500 µl of capture buffer added to the column. The PCR DNA solution was transferred to the column and mixed thoroughly by pipetting up and down 6 times. The column was centrifuged (Eppendorf Centrifuge 5415D) at full speed for 30 seconds (s) and the flow through discarded. The column was placed back into the collection tube, 500 µl of wash buffer added and the column centrifuged at full speed for 30 s. The collection tube was discarded, the column transferred to a sterile microcentrifuge tube and 50 µl of elution buffer (10 mM Tris-HCl, pH 8.0) added directly to the top of the glass fibre matrix of the column. The column was incubated at room temperature (rt) for 1 min and centrifuged at full speed for 1 min to recover the purified DNA.

The DNA concentration was measured in an Eppendorf Biophotometer or stored at -20°C until required for downstream applications.

2.2.3. <u>TRANSFORMATION OF XL-10 GOLD COMPETENT CELLS.</u>

Purified DNA was ligated to the pGEM™ TEasy Vector System I (Promega) in a 10 µl reaction consisting of 1 µl of T4 ligase buffer, 0.2 µl T4 DNA ligase, 3.5 µl insert DNA and 1 µl vector made up to the total volume with sterile Milli-Q water. The ligation mixture was incubated at rt for 2 hours (hrs). One vial of XL-10 gold competent *E. coli* cells (Stratagene) was thawed on ice per ligation to be performed and the entire ligation mixture (10 µl) was added to the thawed cells. The cells were incubated on ice for 30 mins, heat shocked in a 42°C water bath for exactly 40 s and transferred immediately to ice for a further 2 mins. Pre-warmed LB (Luria Bertani) media (800 µl) was added to the cells together with 100 µl of 1 M glucose and 50 µl of 2 M Mg^{2+} and the cells shaken for 1 hr at 37°C. The cells were pelleted by centrifugation in an Eppendorf 5415D at full speed for 30 s and ~900 µl of the supernatant was aspirated. The pellet was resuspended in the remaining 100 µl of supernatant and spread-plated on pre-warmed LB plates supplemented with ampicillin, X-gal (5-Bromo-4-chloro-3-indolyl-A-D-galactopyranoside) and IPTG (Isopropyl-2-D-thiogalactopyranoside). Plates were incubated overnight at 37°C.

2.2.4. <u>ANALYSIS OF TRANSFORMANTS.</u>

White colonies were inoculated into 5 ml LB media supplemented with 50 µl of ampicillin (10 mg.ml^{-1}) and grown overnight on a shaker at 37°C. Plasmid DNA was isolated using the lysis by boiling method.

A 1.5 ml microcentrifuge tube was filled completely with overnight growth and the cells pelleted by centrifugation in an Eppendorf 5415D at full speed for 30 s. The supernatant was aspirated and the pellet was resuspended by vortexing in 350 µl of STET (10 mM Tris, pH 8.0, I mM EDTA, pH 8.0, 0.1 M NaCl, 5% Triton X-100) buffer. Twenty five microlitres of lysozyme (25 mg.ml^{-1}) was added, the tube was vortexed for 3 s and immediately transferred to a boiling water bath for exactly 40 s. The tube was centrifuged at full speed for 10 mins and the cell debris removed with a sterile toothpick. Forty microlitres of sodium acetate (2.5 M $CH_3COO.Na_3.H_2O$, pH 5.2) and 420 µl of isopropanol were added, the tube vortexed and incubated at rt for at least 5 mins to allow DNA precipitation. The tube was centrifuged in an Eppendorf 5417R at full speed for 5 mins at 4°C. The supernatant was aspirated and 1 ml of ice-cold ethanol added. The tube was centrifuged at full speed for 2 mins at 4°C and the

supernatant aspirated. The pellet was dried in a Speedvac Concentrator (SAVANT) for ~10 mins. The pellet was resuspended in 50 µl of TE-RNase buffer and incubated in a 37°C water bath for 30 min to digest contaminating RNA. The DNA was mixed by vortexing and stored at -20°C until required for downstream applications.

Plasmid DNA was purified from solution and analysed by restriction analysis using the restriction endonuclease *Eco*RI for inserts ligated to pBAD/His B and the restriction endonucleases *Eco*RI, *Bam*HI, *Pst*I, *Avr*II and *Bgl*II for inserts ligated to pKOV136. Restriction profiles were observed under UV illumination on 1% agarose gels stained with ethidium bromide.

2.2.5. <u>LIGATION OF COAT PROTEIN GENE INTO VECTOR pBAD/His B.</u>

Plasmid DNA confirmed to have the CP gene insert and the vector pBAD/His B was cleaved separately with the restriction endonucleases *Bgl*II and *Hind*III. The DNA was purified from agarose gels by eluting the CP DNA and vector DNA separately as well as eluting them together with 20 µl of 10 mM Tris (pH 8.0). The CP DNA was then ligated to the vector in two different reactions. One reaction constituted 4 µl of CP DNA, 4 µl of cleaved vector DNA, 1 µl of T4 ligase and 1 µl of T4 ligase buffer. The second reaction consisted of 8 µl of CP DNA and vector DNA that were eluted together, 1 µl of T4 ligase and 1 µl of T4 ligase buffer. The reactions were incubated at rt for 2 hrs and then used to transform competent XL10-Gold *E. coli* cells as described in section 2.2.3. Transformants were analysed and purified from solution but restriction analysis was carried out using double digests with *Bgl*II and *Hind*III to confirm the presence of inserts and with *Bam*HI to confirm orientation of the inserts.

One microlitre of positive plasmid containing the CP gene together with the vector pBAD/His B and pBAD/His/*lacZ* were used to transform competent TOP10 *E. coli* cells as described in section 2.2.3 for the transformation of XL-10 Gold *E. coli* cells. Transformants were analysed and confirmed by restriction analysis with *Bam*HI and double digests with *Bgl*II and *Hind*III. Glycerol stocks of these cultures were made and stored at -20°C.

2.2.6. LIGATION OF COAT PROTEIN GENE INTO VECTOR pKOV136.

Plasmid DNA confirmed to have inserts with the *Acc*651 and *Avr*II or *Bgl*II and *Avr*II sites was cleaved using their respective enzymes while pKOV136 was cleaved using *Acc*651 and *Avr*II or *Bam*HI and *Avr*II in a double digest reaction. The cleaved DNA was purified from an agarose gel and inserts and vector DNA were either eluted separately or together with 20 µl of 10 mM Tris (pH 8.0). The DNA was treated to a number of different techniques to facilitate ligation: insert DNA was completely dried and then ligated to 5 µl of pKOV136 using 1 µl of T4 ligase and 1 µl of T4 ligase buffer in a total reaction volume of 10 µl, the ligation volume was doubled, sequential digestion was performed on the insert DNA and pKOV136 and insert DNA and pKOV136 that was eluted together was dried to 5 µl and then used in a 10 µl reaction volume.

Ligation reactions were used to transform competent XL10-Gold *E. coli* cells as described previously, transformants analysed and confirmed by restriction analysis using a number of restriction endonucleases in single or double digestions. These enzymes were: *Eco*RI, *Acc*651 and *Avr*II, *Pst*I and *Bsm*I and *Avr*II.

2.2.7. BACTERIAL EXPRESSION OF COAT PROTEIN FROM VECTOR pBAD/His B.

Five microlitres of glycerol stock cultures with plasmid DNA containing the CP gene (henceforth referred to as pBAD/His/BFDV CP), pBAD/His B and pBAD/His/*lacZ* was inoculated into 5 ml of LB broth containing 50 µg.ml^{-1} ampicillin and grown overnight on a shaker at 37°C to an OD_{600}=1-2. The next day, 2 tubes each of 5 ml LB broth containing 50 µg.ml^{-1} ampicillin was inoculated with 50 µl of overnight culture of pBAD/His/BFDV CP, pBAD/His B and pBAD/His/*lacZ* and the cultures grown at 37°C with shaking to an OD_{600}=~0.5. A 1 ml sample of cells was removed from each tube, centrifuged at maximum speed (Eppendorf 5415D) for 30 s and the supernatant aspirated. The cell pellet was frozen at -20°C and formed the zero time point sample. A volume of 50 µl from 2% and 0.2% L-arabinose stock solutions was added to one tube each for pBAD/His/BFDV CP, pBAD/His B and pBAD/His/*lacZ*. The cultures were grown at 37°C with shaking for 4 hrs after which 1 ml samples were again removed and pelleted and formed the 4 hr time point sample.

Samples were analysed on 10% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels stained with coomassie blue stain or PageBlue™ Protein Staining Solution (Fermentas). Alternatively, protein bands were detected using the SuperSignal® West HisProbe™ Kit (PIERCE) and expression parameters were optimised further. Optimisation parameters involved using L-arabinose in final concentrations of 0.002%, 0.02%, 0.03% and 0.2%, growth temperatures of 25°C and 37°C and sample points of 1 hr, 3hrs, 6 hrs and 8 hrs post-induction. Following optimisation, the parameters used for bacterial expression of CP included growth temperature of 37°C, induction with a final concentration of 0.2% L-arabinose and cells were harvested 6 hrs post-induction.

2.2.8. EUKARYOTIC (YEAST) EXPRESSION OF COAT PROTEIN FROM VECTOR pKOV136.

Plasmid DNA containing CP gene inserts (henceforth referred to as pKOV136/BFDV CP) was cleaved with the restriction endonuclease *Not*I to linearise the plasmid and allow homologous recombination in *Y. lipolytica* Po1g competent cells. The DNA was purified from an agarose gel as previously described and eluted with 50 μl of 10 mM Tris (pH 8.0). The DNA was used to transform Po1g competent *Y. lipolytica* cells (a W29 derivative).

A glycerol stock of *Y. lipolytica* strain Po1g was streaked out onto a YPD (Yeast Potato Dextrose) plate and incubated overnight at 30°C. The following day, a single colony was used to inoculate 20 ml of YPD-pH4 media and the culture grown overnight on a shaker at 28°C. The culture was allowed to grow to an $OD_{600}=\sim2$ (for W29 derivatives $1OD_{600}=1 \times 10^7$ cells/ml) and the cells were pelleted by centrifuging in a Beckman Model J2-21 centrifuge (JA-20 rotor) at ambient temperature for 5 mins at 5000 X *g*. The supernatant was aspirated and the cells washed with 10 ml of sterile TE buffer (pH 8.0) by resuspending the cells softly with a pipette. The cells were centrifuged at ambient temperature for 5 mins at 3000 X *g* and the supernatant aspirated. Cells were resuspended to a concentration of 5×10^7 cells/ml in 0.1 M LiAc (pH 6.0), transferred to a sterile Erlenmeyer flask and incubated at 28°C with soft shaking (140 revolutions per minute [rpm]) for 1 hr. The cells were pelleted by centrifuging at ambient temperature for 5 mins at 2500 X *g* and the supernatant aspirated. Cells were softly resuspended to a concentration of 5×10^8 cells/ml in

0.1 M LiAc (pH 6.0). Aliquots of 100 µl each of Po1g competent *Y. lipolytica* cells was made and used immediately for transformation.

In a sterile 2 ml microcentrifuge tube, 5 µl of salmon sperm carrier DNA (Sigma) and 5 µl of linearised pKOV136/BFDV CP were added together with 100 µl of Po1g competent *Y. lipolytica* cells. The contents were mixed gently by pipetting, and incubated for 15 mins in a 28°C water bath (without shaking). Thereafter 700 µl of PEG4000 at 40% in 0.1 M LiAc (pH 6.0) was added and mixed gently. The contents were incubated at 28°C for 1 hr with mild shaking (200-250 rpm). The cells were heat shocked for 10 mins in a 39°C water bath; 1 ml of 0.1 M LiAc (pH 6.0) added and mixed gently. A total volume of 300 µl of transformation reaction was streaked out onto selective YNB N$_{5000}$ (Yeast Nitrogen Base without amino acids and ammonium sulphate [1.7 g.L^{-1}], ammonium sulphate [5 g.L^{-1}], glucose [10 g.L^{-1}] and agar [15 g.L^{-1}]) plates. The remaining cells were pelleted by centrifuging in an Eppendorf 5415D for 5 mins at 2500 X *g*, the supernatant was aspirated and the cells resuspended in 300 µl of 0.1 M LiAc (pH 6.0). These cells were plated out on a second selective YNB N$_{5000}$ plate. The plates were incubated at 30°C for at least 2-3 days or until colonies were observed. Forty colonies were transferred to a second YNB N$_{5000}$ plate by streaking out as shown in Figure 2.2.1 A. The plate was incubated at 30°C for 2 days, and then colonies were streaked out on YPD plates as shown in Figure 2.2.1 B.
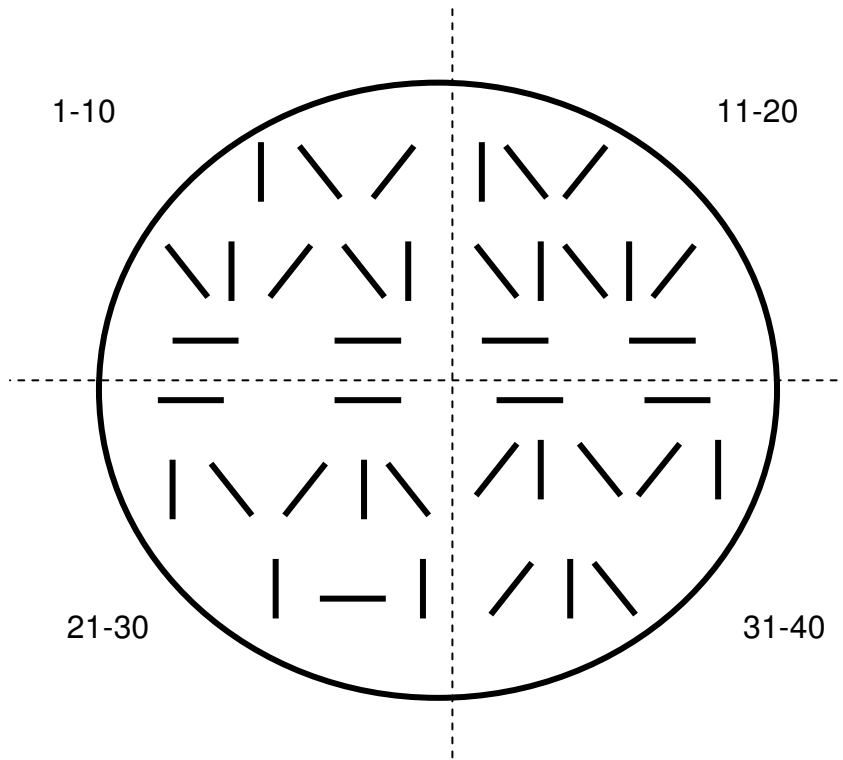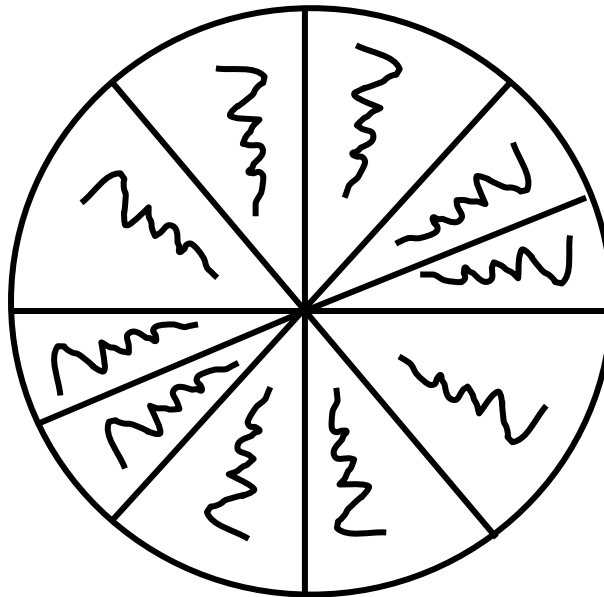
Figure 2.2.1: Plate layout for transferring yeast transformants. A represents the layout for the first transfer and B represents the layout for the second transfer.

Transformants were selected and inoculated into 50 ml of YPD media. The cultures were grown overnight on a shaker at 28°C. The cells were harvested the following day by centrifugation at 4000 X g in a Beckman Model J2-21 centrifuge (JA-20 rotor) at rt. The supernatant was aspirated and the cells weighed. A mass of 500 mg of

yeast cells was used for detection of protein on 10% SDS-PAGE gels and unused cell pellets were stored at -20°C. Gels were either stained with PageBlue™ Protein Staining Solution or used for western blotting. Protein bands were detected on the blots by chemiluminescence using the SuperSignal® West HisProbe™ Kit (PIERCE).

2.2.9. HARVEST OF EXPRESSED PROTEINS.

Bacterial pellets were lysed using a TE lysis buffer (TE containing 1% SDS) by adding 100 µl of lysis buffer to thawed pellets and resuspending by vortexing. The cells were sonicated on ice for 10 s and centrifuged at 20 800 X g for 1 min at 4°C. The supernatant was transferred to a clean microcentrifuge tube and placed on ice together with the pellets. Two hundred microlitres of Laemmli buffer containing ß-mercaptoethanol in the ratio 19:1 was added to the pellets and 100 µl was added to the supernatant. Alternatively, thawed pellets were resuspended in 200 µl of B-PER® Bacterial Protein Extraction Reagent [in phosphate buffer] (PIERCE) by vortexing and shaken for 30 mins at rt. The samples were centrifuged at 16 000 X g for 5 mins and the supernatant transferred to a clean microcentrifuge tube. The pellet was resuspended in 300 µl of B-PER® Bacterial Protein Extraction Reagent (PIERCE) and 10-15 µl of resuspended pellet and supernatant was used to load the gels after adding equal volumes of Laemmli buffer containing ß-mercaptoethanol. Samples were boiled for 10 mins and loaded onto a 10% SDS-PAGE gel (section 2.2.13, chapter 2) together with 5 µl of PageRuler™ Prestained Protein Ladder (Fermentas).

Yeast pellets (500 mg) were resuspended in 1.875 ml of Y-PER® Yeast Protein Extraction Reagent (PIERCE) containing 75 µl of a 25X Complete Protease Inhibitor Cocktail, EDTA-free (Roche) by pipetting up and down. The contents were agitated for 20 mins at rt and the cell debris pelleted by centrifugation at 14 000 X g for 10 mins. The supernatant was transferred to a clean microcentrifuge tube and stored at -20°C until required for gel analysis. Alternatively, yeast cells were lysed using glass beads. Yeast pellets (500 mg) were resuspended in 1 ml of TE containing 1% SDS in a short test tube to which glass beads were added to the bottom of the meniscus. The tubes were vortexed for 30 s and then placed on ice for 1 min. This step was repeated 4 times. Cell debris was pelleted by centrifugation at 14 000 X g for 10 mins. The supernatant was transferred to a clean microcentrifuge tube and stored at -20°C.

2.2.10. POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTS.

Samples between 10 and 15 µl were aliquoted and equal volumes of Laemmli buffer containing ß-mercaptoethanol in the ratio 19:1 was added. Samples were also precipitated using trichloroacetic acid (TCA) precipitation. A volume of 500 µl of 60% w/v TCA was added per 1 ml of protein sample and the sample left on ice for 20 mins. The samples were centrifuged at 10 000 X g for 15 mins at 4°C. The pellet was rinsed with 1 ml of ice-cold propanol and centrifuged at 10 000 X g for 5 mins at 4°C. The supernatant was aspirated and the pellet resuspended in Laemmli buffer containing ß-mercaptoethanol. The protein samples were incubated overnight at 4°C. All the samples were boiled for 10 mins and loaded on 10% SDS-PAGE gels (Table 2.2). A maximum volume of 25 µl per sample and 5 µl of PageRuler™ Prestained Protein Ladder (Fermentas) was loaded. The gels were cast and run in a Mini-PROTEAN 3 Cell System (BIORAD) at a constant current of 20 milliamperes (mA). Gels were either stained overnight with PageBlue™ Protein Staining Solution (Fermentas) or blotted overnight.

Table 2.2: Volumes of each component for 10% resolving and stacking SDS-PAGE gels.

	Resolving (ml)	Stacking (ml)
Distilled water	1.9815	2.441
Sample buffer	1.665	1.615
(Resolving = 1.125 M Tris, pH8.8; 0.3% SDS)		
(Stacking = 0.375 M Tris, pH 6.8; 0.3% SDS)		
Acrylamide/Bisacrylamide (40%, 29:1)	1.25	0.625
Ammonium persulphate (APS, 10%)	50 µl	165 µl
TEMED	10 µl	5 µl

Western blotting was carried out in a Mini Trans-Blot Cell (BIORAD) after pre-washing the gel in transfer buffer (25 mM Tris, pH 8.3; 192 mM glycine, 20% methanol, 0.025-0.1% SDS) for 1 hr. Protein was transferred overnight at 4°C to a HyBond ECL Nitrocellulose Membrane (GE Healthcare) at a constant current of 20 mA. Protein bands on the Western blots were detected by chemiluminescence using the SuperSignal$^®$ West HisProbe™ Kit (PIERCE) or using an anti-rabbit-horseradish peroxidase (anti-rabbit-HRP) antibody.

2.2.11. <u>CHEMILUMINESCENT DETECTION USING SuperSignal® West HisProbe™ KIT.</u>

Protein expressed in frame with a 6XHis tag were detected using the SuperSignal® West HisProbe™ kit (PIERCE) that allows detection of polyhistidine tags independent of their location and adjacent sequences. The nitrocellulose membrane was rinsed in TBST (Tris Buffered Saline Tween®-20) for a few minutes then blocked in 10 ml of BSA/TBST for 1 hr at rt with shaking. It was washed twice on a shaker with 15 ml of TBST for 10 mins each then incubated with 10 ml of HisProbe™-HRP Working Solution for 1 hr at rt with shaking. The membrane was washed 4 times on a shaker with 15 ml of TBST for 10 mins each. The blot was incubated with 10 ml of SuperSignal® West Pico Substrate Working Solution for 5 mins. The blot was placed in a membrane sheet protector which was placed in a film cassette and exposed for 60 s. The film was developed and the blot re-exposed for a number of time lengths.

2.2.12. <u>COLORIMETRIC DETECTION USING Anti-rabbit-HRP/AP ANTIBODY.</u>

The nitrocellulose membrane was rinsed in phosphate-buffered saline with 0.05% Tween 20 (PBST) at rt with shaking for a few mins. The membrane was blocked in 10 ml PBST containing 5% non-fat, dry milk powder (blocking buffer) for 1 hr at rt with shaking. It was washed in 20 ml PBST twice for 5 mins each with agitation. The membrane was transferred to another tray containing 10 ml of rabbit raised anti-BFDV diluted 1:1000 in blocking buffer and incubated with shaking at rt for 1 hr. The membrane was washed as in the previous wash step and transferred to a tray containing 10 ml of anti-rabbit-HRP diluted 1:10 000 in blocking buffer and incubated at rt for 1 hr with shaking. The membrane was washed as described previously and transferred to a tray containing 10 ml of SuperSignal® West Pico Substrate Working Solution (PIERCE) for 5 mins. The membrane was then exposed to film as described in the preceding section (2.2.10).

Colorimetric detection using an anti-rabbit-alkaline phosphatase (anti-rabbit-AP) was also performed. The same protocol was followed as described above for the chemiluminescent detection using anti-rabbit-HRP but the substrate used was *p*-nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) and the

membrane was incubated in the substrate overnight on a shaker at rt until blue/purple protein bands were observed.

2.2.13. <u>PURIFICATION OF EXPRESSED BACTERIAL PROTEIN.</u>

Harvested bacterial cells were resuspended in 100 mM MOPS buffer (3-morpholinopropanesulfonic acid, pH 7.4) containing 0.5 M NaCl and 20 mM imidazole and the cells broken by ultrasonic treatment in 1 min bursts at 80%. The cells were exposed to the ultrasonic treatment until the lysate reduced in turbidity indicating efficient lysis of the cells. Unbroken cells and debris was removed by centrifugation at 4000 X g for 10 mins and the recombinant CP purified on a nickel chelating column. The supernatant was loaded onto a HisTrap FF column (5 ml, GE Healthcare) and unbound proteins eluted at a flow rate of 5 ml.min^{-1} using 20 mM MOPS buffer (pH 7.4) containing 0.5 M NaCl and 20 mM imidazole. Bound proteins were then eluted in the same buffer using a linear gradient (100 ml) of imidazole up to 500 mM. Fractions suspected to contain recombinant CP were analysed by SDS-PAGE. Fractions identified as having recombinant CP were pooled and dialysed against the elution buffer. One of the pooled samples was reloaded onto the HisTrap FF column and subjected to a second round of purification using 20 mM MOPS buffer (pH 7.4) containing 0.5 M NaCl and 40 mM imidazole. Fractions suspected to contain recombinant CP were analysed using SDS-PAGE.

Expressed bacterial protein was also purified using the B-PER® 6xHis Fusion Protein Purification Kit (PIERCE).The column and buffers were equilibrated to rt whilst the bacterial cells from a 250 ml culture thawed. The bacterial pellet was resuspended in 10 ml of B-PER® Reagent containing 40 µl of 25X Complete Protease Inhibitor Cocktail, EDTA-free (Roche) by vortexing until the cell suspension was homogenous. The soluble proteins were separated by centrifugation at 27 000 X g for 15 mins at rt. The supernatant was transferred to a clean microcentrifuge tube. The Nickel Chelated Column was uncapped and the sodium azide storage solution allowed to drain from the gel bed. The gel bed was prepared by adding 10 ml (2 X 5 ml) of B-PER® Reagent and allowing it to flow through the column. Up to 10 ml of the sample (2 X 5 ml) was applied to the column and allowed to flow through the gel bed. The flow through from each step was collected and analysed using SDS-PAGE. The column was washed with 6 ml (2 X 3 ml) of Wash Buffer 1, allowing it to flow through the column, and then with 9 ml (3 X 3 ml) of Wash Buffer 2. The expressed 6xHis-

tagged protein was eluted by adding 6 ml (2 X 3 ml) of Elution Buffer and the fractions collected. Aliquots of 15 µl from the flow through and the eluted fractions were analysed by SDS-PAGE.

2.2.14. <u>QUANTIFICATION OF PARTIALLY PURIFIED RECOMBINANT COAT PROTEIN USING BCA PROTEIN ASSAY.</u>

Purified bacterial expressed CP was quantified using the Micro BCA™ Protein Assay Reagent Kit (PIERCE) to obtain total protein concentration. Rabbit raised polyclonal anti-BFDV was also quantified. Table 2.3 indicates the guide used to prepare diluted bovine serum albumin (BSA) standards using 50 mM carbonate buffer (pH 9.6) as the diluent as the protein and antibodies was to be used in ELISAs.

Table 2.3: Preparation of diluted albumin standards.

Vial	Volume of diluent (ml)	Volume and source of BSA (ml)	Final BSA concentration ($\mu g.ml^{-1}$)
A	4.5	0.5 of stock	200.0
B	8.0	2.0 of vial A dilution	40.0
C	4.0	4.0 of vial B dilution	20.0
D	4.0	4.0 of vial C dilution	10.0
E	4.0	4.0 of vial D dilution	5.0
F	4.0	4.0 of vial E dilution	2.5
G	4.8	3.2 of vial F dilution	1.0
H	4.0	4.0 of vial G dilution	0.5
I	8.0	0	0 = blank

The following formula was used to determine the volume of Micro BCA™ Working Reagent (WR) required to perform the assay in duplicate:

(9 + 3) (2) (1) = 24

Where:

9 = number of standards

3 = number of unknown samples

2 = number of replicates

1 = the volume of WR in ml required per sample

24 = the total volume of WR required.

Fresh WR was prepared by combining 12.5 ml of Reagent MA and 12.0 ml of Reagent MB with 0.5 ml of Reagent MC (according to the ratio 25:24:1, Reagent MA:MB:MC). One millilitre of each standard and unknown sample was pipetted into a clean and appropriately labelled test tube (this was done in duplicate). One millilitre of WR was added to each tube and mixed well. The tubes were covered and incubated in a 60°C water bath for 1 hr. The tubes were cooled to rt and absorbance readings taken at 562 nm in a spectrophotometer zeroed with water. The average A_{562} reading of the Blank replicates was subtracted from the A_{562} readings of all the other individual standard and unknown sample replicates. A standard curve was plotted using the average Blank-corrected A_{562} readings for each BSA standard versus its concentration in $\mu g.ml^{-1}$ and used to determine the protein concentration of each unknown sample.

2.2.15. <u>INDIRECT ELISA USING EXPRESSED RECOMBINANT COAT PROTEIN.</u>

In the indirect ELISAs, 100 µl of bacterially expressed BFDV CP (5 $\mu g.ml^{-1}$) in 50 mM carbonate buffer (pH 9.6) was used to coat the wells of a Costar polypropylene ELISA plate. A crude lysate of Po1g *Y. lipolytica* cells suspected to have expressed BFDV CP was diluted 1:2 in 50 mM carbonate buffer (pH 9.6) and also used to coat a plate for an indirect ELISA. Background controls were included where wells were coated with 100 µl of 50 mM carbonate buffer (pH 9.6) and the plate was incubated overnight at 4°C. Other controls in separate ELISAs included coating wells with TOP10 *E. coli* cells. The following day the wells were washed three times with 200 µl of PBST. The wells were blocked with 300 µl of PBST containing 10% non-fat, dry milk powder (blocking buffer) for 1 hr at 37°C with shaking. The washing step was repeated and 100 µl of primary antibody (rabbit raised anti-BFDV, chicken raised anti-BFDV CP or rabbit raised anti-BFDV adsorbed to *E. coli* cells) at different dilutions in the blocking buffer was added to the respective wells. The plate was incubated at 37°C with shaking for 1 hr and washed as previously. Secondary antibody (anti-rabbit-HRP/anti-chicken-HRP) was diluted (1:10000/1:30000) in blocking buffer and 100 µl was added to each well. The plate was incubated at 37°C with shaking for 1 hr. Once again the plate was washed as before and 100 µl of substrate (4, 4, 5, 5'-tetramethylbenzidine [TMB]) was added to each well. Colour development was allowed to occur for 10 mins and the reaction was stopped by the addition of 100 µl of 2N H_2SO_4. Absorbance readings were taken at 450 nm.

2.2.16. INDIRECT COMPETITIVE ELISA USING EXPRESSED RECOMBINANT COAT PROTEIN.

The same protocol used for the indirect ELISA in this study was used for the competitive ELISA with minor modifications. A volume of 100 µl of non-purified or partially purified bacterially expressed recombinant CP (5 µg.ml^{-1}) in 50 mM carbonate buffer (pH 9.6) was used to coat the wells of a Costar polypropylene ELISA plate. Background controls included coating the wells with 50 mM carbonate buffer (pH 9.6) and the plate was incubated overnight at 4°C. The following day the wells were washed three times with 200 µl of PBST and the wells blocked with 300 µl of PBST containing 10% non-fat, dry milk powder (blocking buffer) for 90 mins at 37°C with shaking. The wells were then washed three times with 200 µl of PBST followed by the addition of primary antibody which consisted of various dilutions of parrot antibodies or fetal bovine serum (FBS) added together with rabbit raised anti-BFDV diluted 1:100 or parrot antibodies and rabbit raised anti-BFDV added individually. Each serum was tested in four separate wells. The plate was incubated at 37°C for 1 hr with shaking and then washed three times with 100 µl of PBST. Secondary antibody (anti-rabbit-HRP) was diluted 1:10000 in blocking buffer and 50 µl was added to each well. The plate was incubated at 37°C with shaking for 1 hr, washed three times with 100 µl of PBST and 50 µl of substrate TMB was added to each well. Colour development was allowed to occur for 10 mins and the reaction was stopped by the addition of 50 µl of 2N H$_2$SO$_4$. Absorbance readings were taken at 450 nm.

2.2.17. USE OF ANTI-CHICKEN ANTIBODIES TO DETECT PARROT ANTIBODIES.

A simple test was carried out in order to determine if anti-chicken antibodies can bind to parrot raised antibodies and detect them as there are no commercially available anti-parrot conjugated antibodies for use in an indirect ELISA. As *Newcastle disease virus* (NDV) does not infect parrots, nine parrots housed at the animal facility at the UFS were intranasally vaccinated with the La Sota NDV vaccine (Animal experiment number 15/02). Blood was collected pre-inoculation and post inoculation after 7, 14, 21, 35 and 49 days and the sera stored at -20°C until required. Sera were tested for the presence of anti-NDV antibodies using the FlockChek Newcastle Disease Virus Antibody Test (Idexx) according to the manufacturer's protocol.

The reagents were allowed to come to rt and mixed gently by inverting and swirling. A volume of 100 µl of undiluted negative control was added into wells A1 and A2 and 100 µl of undiluted positive control was added into wells A3 and A4. The sera samples were diluted 1:500 with sample diluent and 100 µl of each sample was added into appropriate wells. Each sample was tested in duplicate. The ELISA plate was incubated at rt for 30 mins, the liquid of all wells aspirated and each well washed three times with 200 µl of distilled water. The water was aspirated completely after each wash. A volume of 100 µl of (Goat) Anti-Chicken: Horseradish Peroxidase Conjugate was added to each well and the plate incubated at rt for 30 mins. The washing step was repeated and 100 µl of TMB substrate solution was added into each well. The plate was incubated for 15 mins at rt and 100 µl of Stop Solution was dispensed into each well to stop the reaction. The reader was blanked and absorbance readings at 620 nm were taken.

The sera was also tested using the HA and HI assays as described by Villegas (1989) with some modification. A volume of 50 µl of PBS buffer containing calcium and magnesium ions was added to each well. For the HA test, a volume of 50 µl of NDV (antigen) was added to the first well and two-fold serially diluted across the plate. A volume of 50 µl of 0.5% w/v chicken (*Gallus gallus*) red blood cells (RBCs) was added and the plate incubated at rt for 60 mins. The end point dilution where a lattice formed was noted and used to calculate the dilution of the antigen which would result in 4 haemagglutination units (HAU) which were used in the HI assay. For the HI assay, a volume of 50 µl of PBS buffer containing calcium and magnesium ions was added to each well followed by the addition of 50 µl of serum to the first well. The serum was two-fold serially diluted across the plate after which 50 µl of 4 HAU of NDV was added to each well. The plate was incubated at rt for 30 mins. Thereafter, 50 µl of 0.5% w/v chicken RBCs was added to each well and the plate incubated for a further 60 mins. HI readings were taken as the last well to have a button formed before haemagglutination was observed. A graph of the correlation between the ELISA readings and the HI readings was constructed using the data collected in the two assays.

2.2.18. <u>SUMMARY OF EXPERIMENTAL DESIGN.</u>

A brief overview of all the major steps carried out during the expression of recombinant CP in *E. coli* and in *Y. lipolytica* is outlined in Figure 2.2.2.
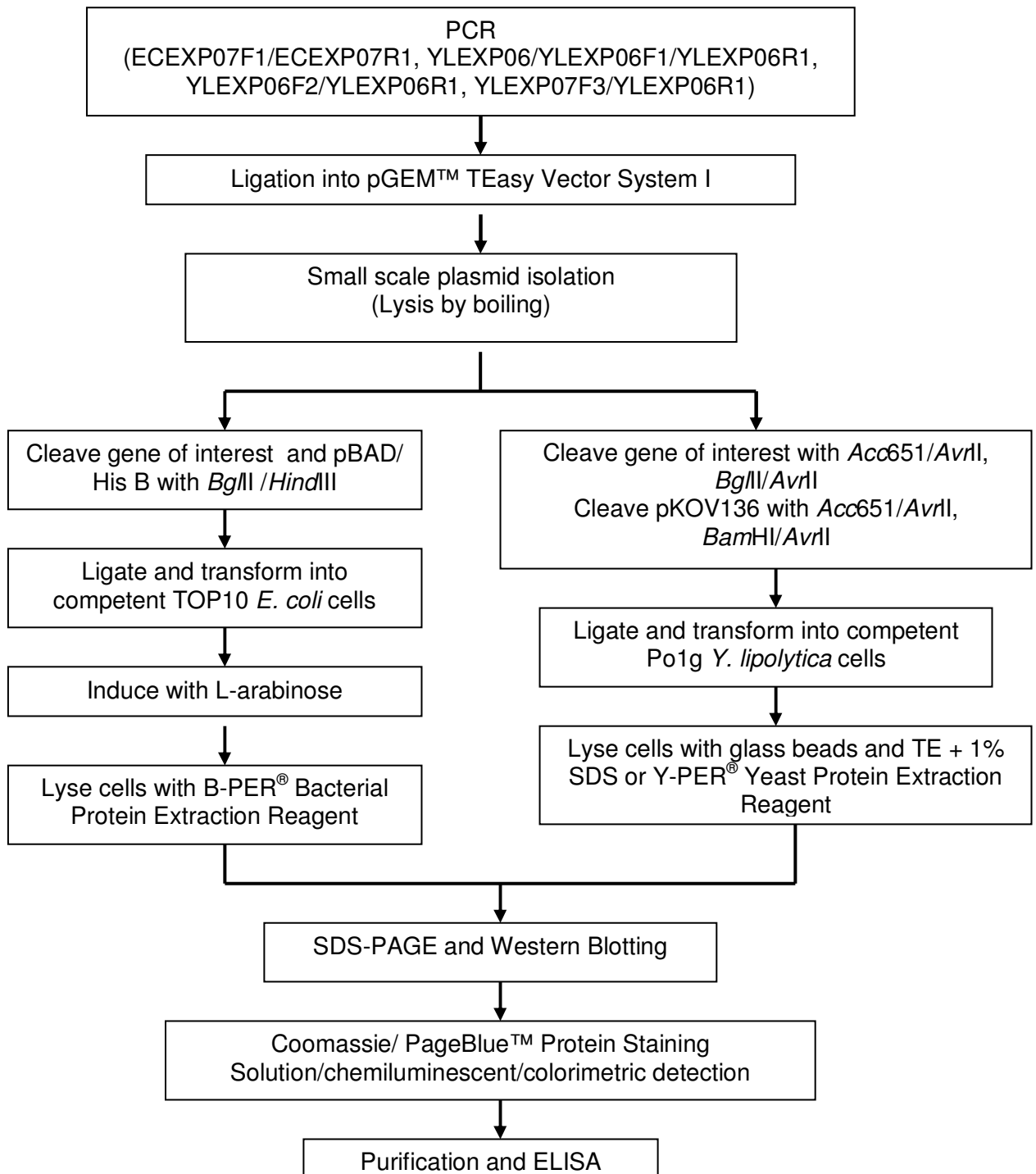
Figure 2.2.2: Methods used for bacterial and yeast protein expression.

2.3. [A] RESULTS AND DISCUSSION FOR EUKARYOTIC (YEAST) EXPRESSION.

2.3.1. PCR AND RESTRICTION PROFILES FROM PRIMER SET YLEXP06 F1/ YLEXP06 R1.

Using a BFDV sequence deposited in GenBank, Accession Number AF080560, as the DNA template for primer design, PCR amplification of the CP gene using the primer set YLEXP06 F1 and YLEXP06 R1 was expected to yield a fragment of 774 base pairs (bp). Figure 2.3.1 A indicates the fragments obtained from PCR amplification. These PCR products were ligated into the vector pGEM™ TEasy Vector System I (Promega) and the presence of inserts in isolated plasmid DNA was confirmed in a double digest using *Acc*65I and *Avr*II [Figure 2.3.1 B].
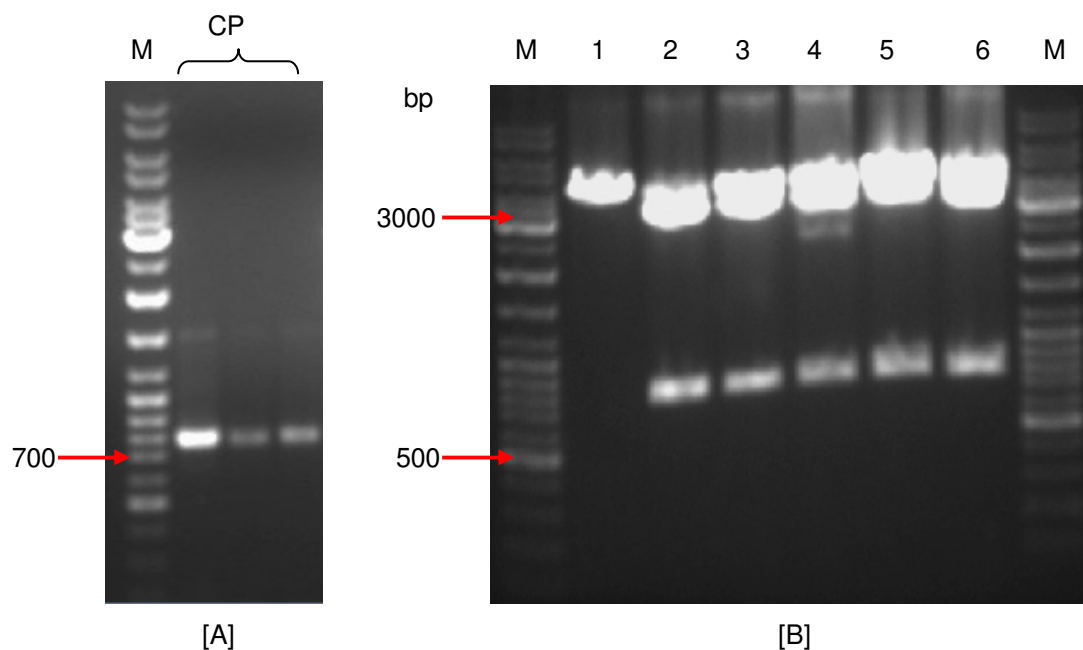
Figure 2.3.1: A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the CP gene of BFDV using the primer set YLEXP06 F1/YLEXP06 R1 [A] and the presence of inserts in pGEM™ TEasy Vector System I [B]. Lane 1 did not have an insert with an *Acc*65I and *Avr*II recognition site while lanes 2-6 yielded inserts of the expected size (~770 bp) when cleaved with *Acc*65I and *Avr*II. Lane M represents the molecular weight marker, O' GeneRuler™ DNA Ladder Mix (Fermentas).

A number of attempts to ligate the CP inserts into the vector pKOV136 were unsuccessful. The vector pKOV136 was then cleaved with *Acc*65I to confirm the presence of its recognition site. Figure 2.3.2 confirms the presence of the *Acc*65I recognition site which indicated that the plasmid was still functional for ligation using this recognition site. However, repeated ligations once again did not yield any positive results.
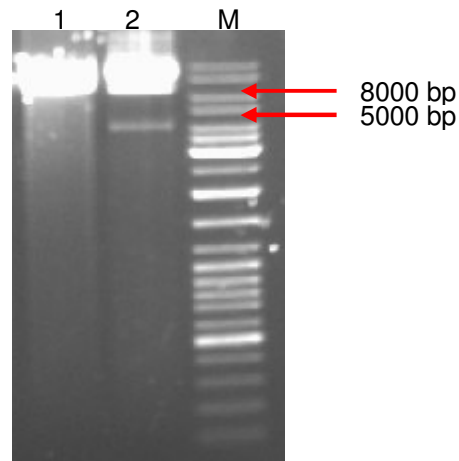
Figure 2.3.2: A 1% w/v agarose gel visualised under UV illumination confirming the presence of the *Acc*65I recognition site in the vector pKOV136. Lane 1 indicates plasmid cleaved with *Acc*65I yielding linearised plasmid of 6578 bp. Lane 2 indicates the expected profile of the circular nicked, linear and supercoiled forms for uncut plasmid pKOV136. Lane M represents the molecular weight marker, O' GeneRuler™ DNA Ladder Mix (Fermentas).

Isolated plasmid DNA was also cleaved with the restriction endonucleases *Bam*HI and *Pst*I but no inserts were observed. It was then decided to redesign the forward primer to use the *Bam*HI site on pKOV136 for ligation into the vector by designing the forward primer YLEXP06 F2 to include a *Bgl*II recognition site. *Bam*HI cleaves the CP gene into two fragments and so can not be used for ligation but *Bgl*II is a non-cutter of the gene and forms compatible sticky ends with *Bam*HI allowing ligation of the CP gene to pKOV136 using this recognition site.

2.3.2. PCR AND RESTRICTION PROFILES FROM PRIMER SET YLEXP06 F2/ YLEXP06 R1.

PCR amplification yielded fragments of ~800 bp as well as a non-specific fragment of ~500 bp [Figure 2.3.3 A], but when cleaved with *Bam*HI, only the expected fragment

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of ~800 bp was cleaved to yield two fragments of ~ 200 bp and ~600 bp [Figure 2.3.3 B]. *Bam*HI cleaves the CP gene template, AF080560, once to yield two fragments of 184 bp and 590 bp hence the 800 bp fragment was purified from an agarose gel and used for ligation reactions.

Figure 2.3.3: A 1% w/v agarose gel visualised under UV illumination showing the PCR products from the primer set YLEXP06 F2/YLEXP06 R1 [A]. Lanes 1-3 are positively amplified fragments of ~800 bp and a non-specific amplified fragment of ~500 bp. Lane – is a negative control. Figure [B] indicates the RFLP profile obtained when the PCR products were cleaved with the restriction endonuclease *Bam*HI; *Bam*HI cleaved the ~800 bp fragment into two fragments ~200 bp and ~600 bp while the ~500 bp fragment was not cleaved (Lane 1). Lane – is a negative control. Lanes M represents the molecular weight marker, O' GeneRuler™ DNA Ladder Mix (Fermentas).

Plasmid isolations of pGEM™ TEasy Vector containing CP inserts were confirmed by cleavage with *Eco*RI but the profiles that were observed as seen in Figure 2.3.4 were different from the expected profile as *Eco*RI does not cleave within the CP insert but removes the entire insert from pGEM™ TEasy Vector I. It was presumed that the different restriction fragment length polymorphism (RFLP) profile was obtained because the PCR product was not the CP gene due to the presence of the non-specific fragment in the PCR amplification and therefore another set of YLEXP06 F2 and YLEXP06 R1 primers were ordered from a different company.
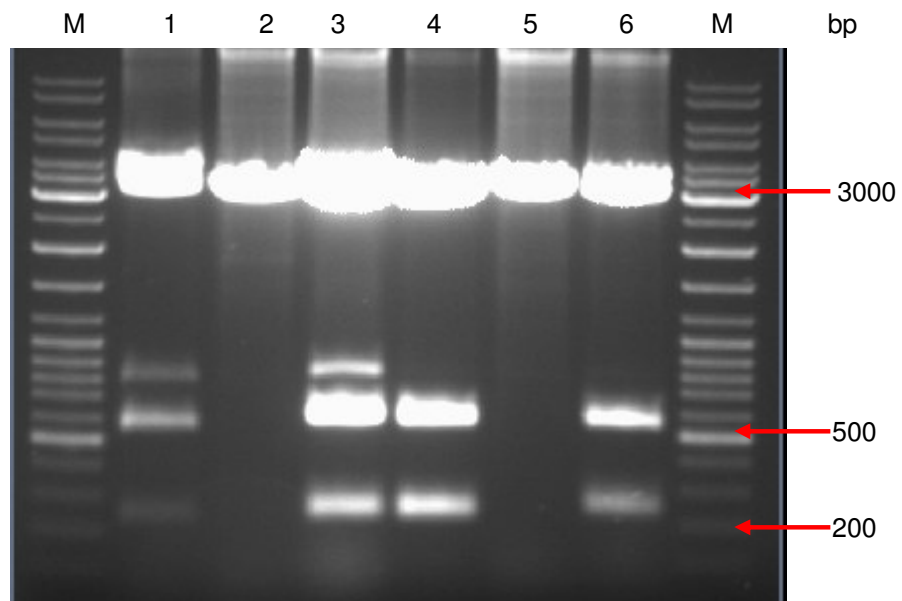
Figure 2.3.4: A 1% w/v agarose gel visualised under UV illumination indicating an unexpected RFLP profile for pGEM™ TEasy Vector containing CP gene inserts. Lane M represents the molecular weight marker, O' GeneRuler™ DNA Ladder Mix (Fermentas).

PCR using the second set of primers YLEXP06 F2 and YLEXP06 R1 yielded a single fragment of ~800 bp [Figure 2.3.5 A]. However, when the PCR product was cleaved with the restriction endonucleases *Bam*HI, *Pst*I and *Dpn*I in separate reactions to confirm that the product was indeed the CP gene, only the RFLP profile obtained with *Bam*HI was the expected profile while the other profiles did not conform to the expected profiles [Figure 2.3.5 B].
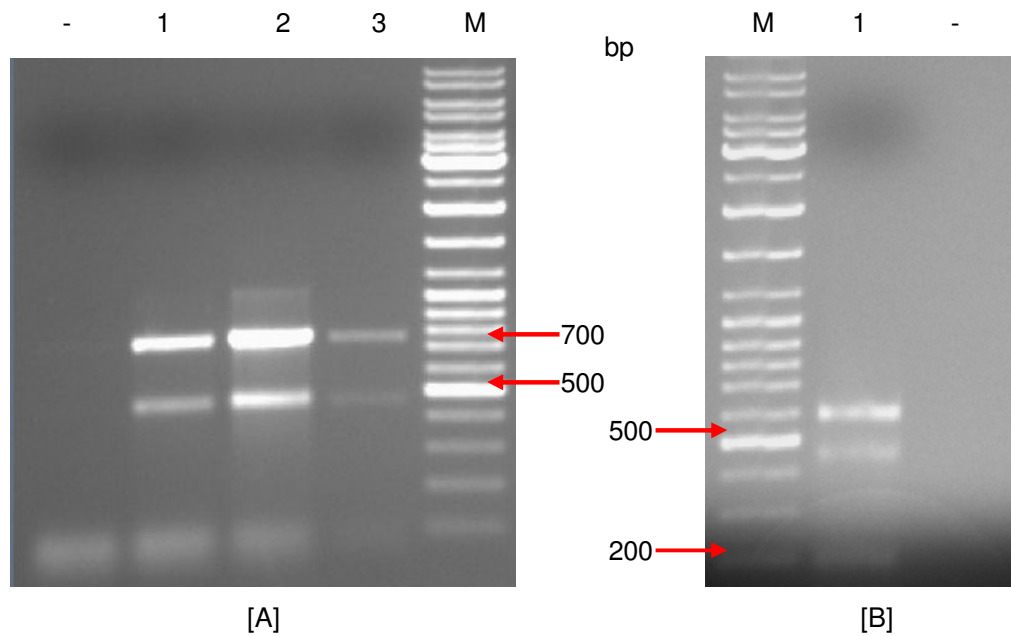
Figure 2.3.5: A 1% w/v agarose gel visualised under UV illumination showing the PCR products (~800 bp) from the primer set YLEXP06 F2/YLEXP06 R1 purchased from a second company [A]. Figure [B] indicates the RFLP profiles obtained from cleaving the PCR product with *Bam*HI (lane 1), *Pst*I (lane 2) and *Dpn*I (lane 3). Only cleavage with *Bam*HI produced the expected RFLP profile of 184 bp and 590 bp while *Pst*I and *Dpn*I gave unexpected profiles. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

Uncertainty regarding these unexpected profiles led to the decision to amplify the CP gene from a larger portion of the BFDV genome. Primers used in a previous study at the University of the Free State (UFS), PBF F1 (5'-AACCCTACAGACGGCGAG3', position 182-199) and PBF R1 (5'-GTCACAGTCCTCCTTGTACC'3', position 879-898), were designed to amplify the entire Rep gene from BFDV. Using different combinations of these primers with the primers designed in the current study, the majority of the BFDV genome could be amplified.

The primer combinations PBF F1/YLEXP06 F1, PBF F1/YLEXP06 F2 and PBF R1/YLEXP06 R1 were used to amplify large portions of the BFDV genome of the expected sizes of 1802 bp, 1802 bp and 1680 bp, respectively. Figure 2.3.6 A shows the two fragments amplified using the primer combinations PBF F1/YLEXP06 F1 and PBF R1/YLEXP06 R1 and Figure 2.3.6 B indicates their respective RFLP profiles when cleaved with the restriction endonucleases *Bam*HI and *Pst*I. No PCR product was amplified from primer combination PBF F1/YLEXP06 F2.
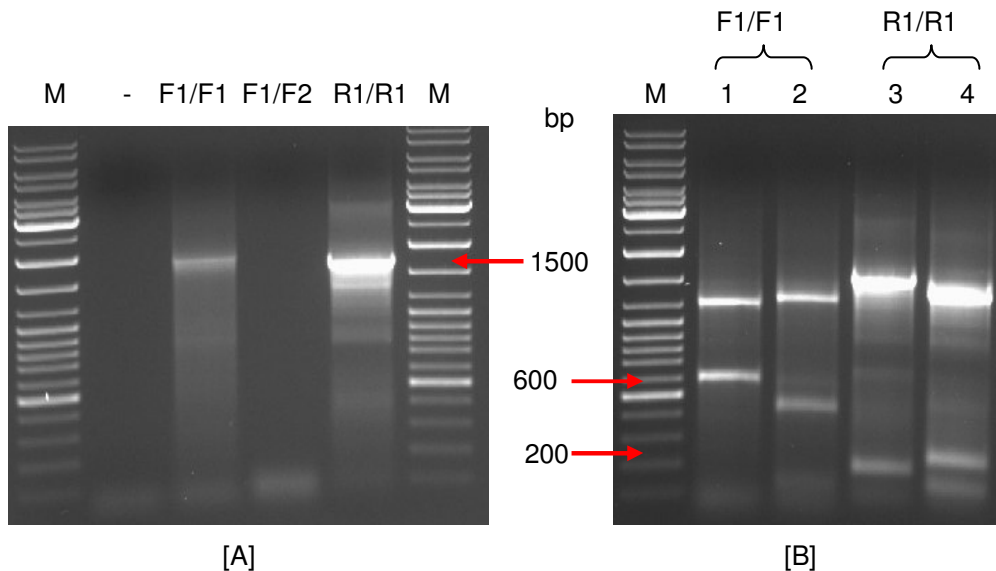
Figure 2.3.6: A 1% w/v agarose gel visualised under UV illumination showing the PCR products from the primer set PBF F1/YLEXP06 F1 (lane F1/F1), PBF F1/YLEXP06 F2 (lane F1/F2) and P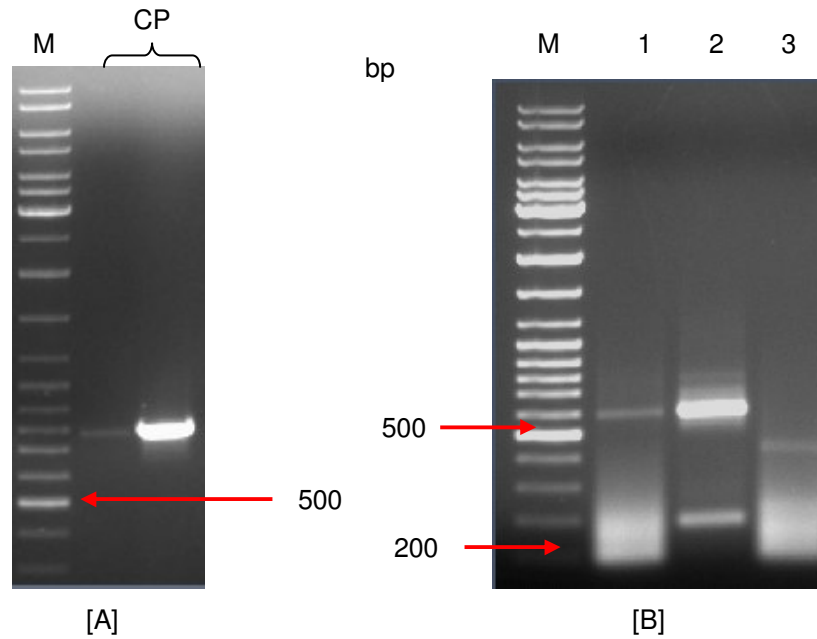BF R1/YLEXP06 R1 [A]. Figure [B] indicates the RFLP profiles of PCR products from primer combinations PBF F1/YLEXP06 F1 and PBF R1/YLEXP06 R1 that were cleaved separately with *Bam*HI and *Pst*I (lanes 1 & 3 for *Bam*HI and lanes 2 & 4 for *Pst*I), respectively. The expected profiles for *Bam*HI and *Pst*I for PBF F1/YLEXP06 F1 were observed but only the expected profile for *Bam*HI for PBF R1/YLEXP06 R1 was seen while the profile for *Pst*I was not the predicted profile. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

The expected profile using *Bam*HI was obtained [Figure 2.3.6 B] and included two fragments of 593 bp and 1209 bp for the combination PBF F1/YLEXP06 F1 and 180 bp and 1500 bp for the combination PBF R1/YLEXP06 R1. An expected profile of 421 bp and 1381 bp for combination PBF F1/YLEXP06 F1 was also observed when *Pst*I was used for cleavage but the expected profile of 352 bp and 1328 bp for combination PBF R1/YLEXP06 R1 was not observed but instead three fragments ~1200, 300 and 200 bp were seen [Figure 2.3.6 B]. The PCR products from Lanes F1/F1 and R1/R1 in Figure 2.3.6 A were ligated into pGEM™ TEasy Vector System I and plasmid isolations cleaved with *Eco*RI to confirm the presence of inserts. Based on the DNA template used for primer design, the expected CP gene is not cleaved by *Eco*RI for either of the primer combinations and so should remove the insert from the pGEM™ TEasy backbone. This was observed as indicated in Figure 2.3.7 A & B for some of the plasmid isolations.
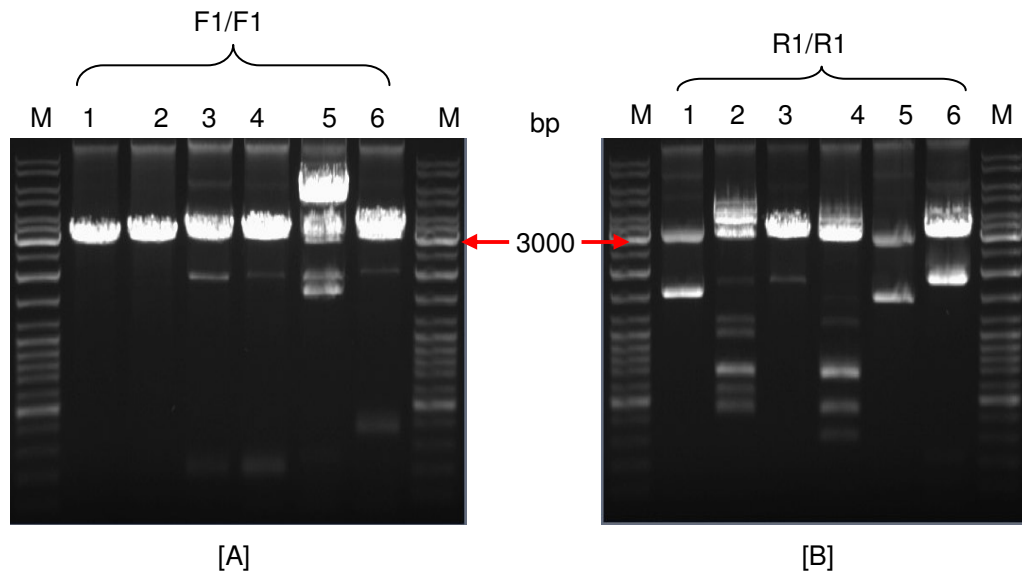
Figure 2.3.7: A 1% w/v agarose gel visualised under UV illumination indicating possible inserts in select plasmid isolations from transformations of competent *E. coli* with PCR products from PBF F1/YLEXP06 F1 and PBF R1/YLEXP06 R1 ligated to pGEM™ TEasy Vector I. Plasmid isolates were cleaved with *Eco*RI to remove inserts of 1802 bp [A] and 1680 bp [B] from the 3015 bp pGEM™ TEasy Vector backbone. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

Some of the plasmid DNA clones (lanes 3 and 4 in Figure 2.3.7 A and lanes 1 and 5 in Figure 2.3.7 B) were further cleaved using double digestion with a combination of *Eco*RI and *Bam*HI and *Eco*RI and *Pst*I. The expected profiles were not observed for products from either the combination PBF F1/YLEXP06 F1 [Figure 2.3.8 A] or combination PBF R1/YLEXP06 R1 [Figure 2.3.8 B].
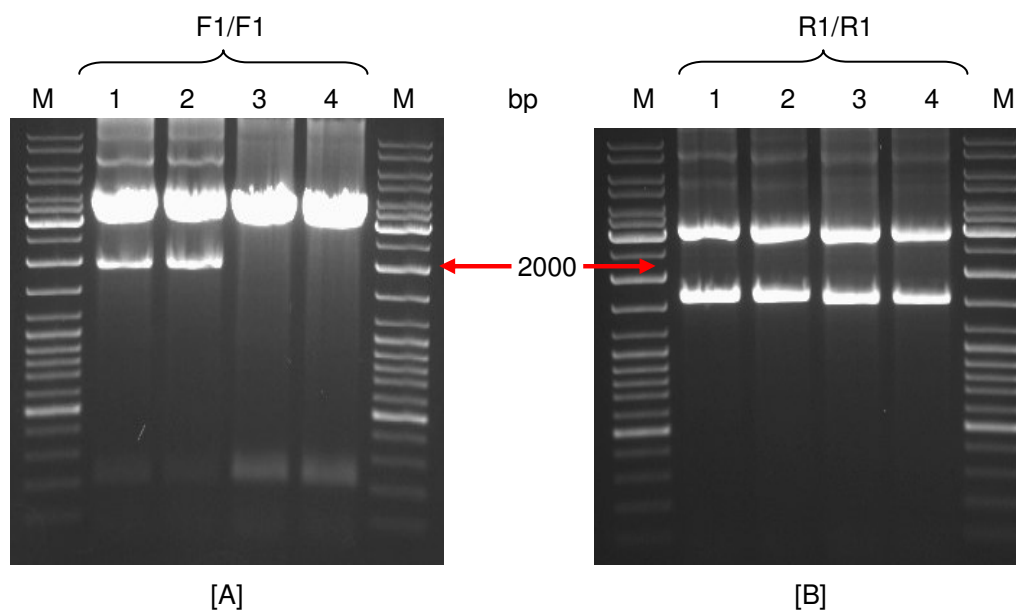
Figure 2.3.8: A 1% w/v agarose gel visualised under UV illumination indicating RFLP profiles obtained for plasmid isolates of PBF F1/YLEXP06 F1 [A] and PBF R1/YLEXP06 R1 [B] ligated to pGEM™ TEasy Vector I from a double digest using *Eco*RI/*Bam*HI and *Eco*RI/*Pst*I. Lanes 1 and 3 represent the *Eco*RI/*Bam*HI profile and lanes 2 and 4 represent the *Eco*RI/*Pst*I profile. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

The PCR amplification was repeated using the primer combinations PBF F1/YLEXP06 F1 and PBF R1/YLEXP06 R1, products were ligated into pGEM™ TEasy Vector I and small scale plasmid isolations performed. The isolated plasmid DNA and PCR product were cleaved with *Eco*RI and two fragments were observed in the cleaved PCR product of ~300 bp and ~1500 bp. The same two fragments in addition to the 3015 bp backbone of pGEM™ TEasy Vector were also observed in some of the isolated plasmid DNA as indicated in Figure 2.3.9 (lanes 1 and 4). These profiles were unexpected as the DNA template AF080560 is not cleaved by *Eco*RI. Plasmid isolates 1 and 4 (lanes 1 and 4 in Figure 2.3.9) were sent for sequencing to Inqaba Biotechnologies Ltd and sequences were analysed using DNAssist v2.3.
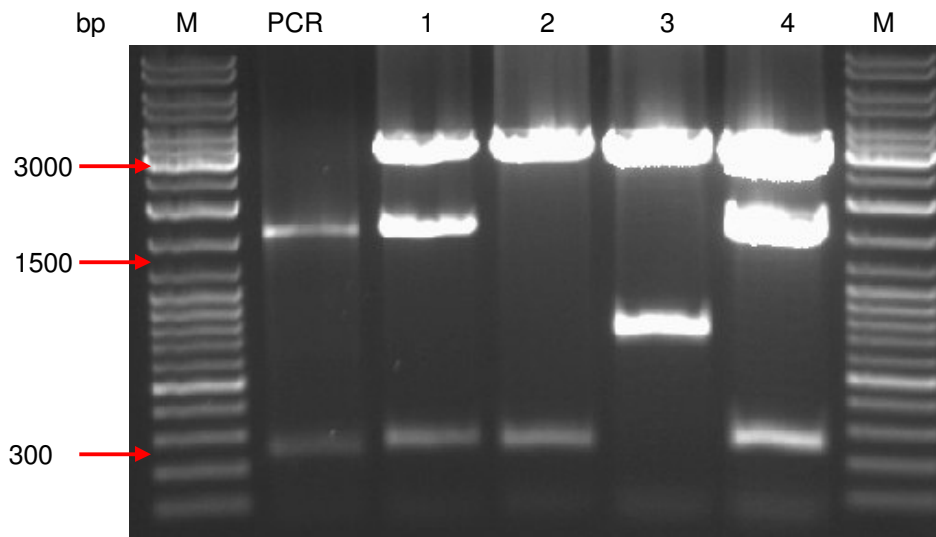
Figure 2.3.9: A 1% w/v agarose gel visualised under UV illumination indicating an unexpected RFLP profile for PCR (lane PCR) and pGEM™ TEasy Vector containing CP gene inserts (lanes 1-4) after cleavage with *Eco*RI. Lane M represents the molecular weight marker, O' GeneRuler™ DNA Ladder Mix (Fermentas).

2.3.3. SEQUENCING RESULTS FOR PARTIAL BFDV GENOME FROM PBF F1/YLEXP06 F1.

Analysis of the sequences received from Inqaba Biotechnologies Ltd from plasmid isolated DNA 1 and 4 revealed that it was BFDV but the sequence was different from the DNA template AF080560 that was used to design the primers. The entire CP gene sequence is indicated in Figure 2.3.10 while Table 2.4 indicates the expected RFLP profiles when the CP gene (this study) is cleaved using the most common restriction endonucleases used in the present study.

```
1    ctgtggggcacctctaactgcgcatgcgctaaatttcagattagacgacgtagatatgcc 60
61   cgcccatactaccgccgacgtcacatcaggcgataccgccggcgacgtagatacttccgc 120
121  aaacgccgtttctcaaccaataggatttacactctcagattcaaacgccaattaaaattt 180
181  gaaattctgaaacaaactactcaagctggacaagtgatttggaattctgactacataacc 240
241  ttttcattgtcagacttcctagcaacaaacccgaacccccatacactgaatttcgaagac 300
301  taccgtattaaattagctaaaatggaattgaggcccacatggggccactacaccattagt 360
361  gcagacggattcggccacacggccgtaatccaagattccagaataactaaatttaaaact 420
421  actgcagaccaatcacaagacccagtggcacctttcgacggtgccaagaaatggtacctt 480
481  agcaggggattcaaacgcctgctcagaccgaaaccacaaataacaattgatgacctgacc 540
541  acttcgaaccagtctgcagcattgtggctaaacagcgccagaaccggctggatcccatta 600
601  caaggaggacccaacgcggcgggagcgaacgtcaagcactacggccttgtattcagcttc 660
661  cctcagccggaagtaaagatatcttatgtatgcgagctcactctgtatgttcaattccgc 720
721  agtttgccccaaacaatcccagtacttcttaa
```

Figure 2.3.10: Entire CP gene sequence for BFDV isolate used in the present study indicating the presence of restriction endonuclease sites that have been colour coded as follows: *Eco*RI, *Pst*I, *Acc*65I, and *Bam*HI.

Table 2.4: Important restriction endonucleases that cut the CP gene used in the present study.

Enzyme	Recognition site	Cleavage site	Frequency of cuts
*Acc*65I	G▾GTAC▴C	473/477	1
*Bam*HI	G▾GATC▴C	590/594	1
*Eco*RI	G▾AATT▴C	222/226	1
*Pst*I	C▴TGCA▾G	426/422, 558/554	2

*Avr*II (C▾CTAG▴G) and *Bgl*II (A▾GATC▴T) are non-cutters.

Unlike the DNA template AF080560 that was used for the design of primers, the CP gene used in this study is cleaved by both *Eco*RI and *Acc*65I. It is therefore likely that failure for the PCR product to successfully ligate into the vector pKOV136 when using the primer combination YLEXP06 F1/YLEXP06 R1 can be attributed to the presence of the *Acc*65I recognition site. Each time the CP gene was removed from pGEM™ TEasy Vector I by cleaving with *Acc*651 and *Avr*II, *Acc*651 cleaved the CP gene into two fragments preventing ligation into pKOV136. It is also reasonable to suggest that the unexpected RFLP profiles observed when amplified fragments were cleaved with *Eco*RI and *Pst*I was due to *Eco*RI cleaving once within the CP gene and *Pst*I cleaving twice within the CP gene as indicated in Table 2.4. The presence of these differences in the CP gene sequence lends further evidence to the genetic diversity present in BFDV isolates that is frequently attributed to genetic differences in the CP gene.

2.3.4. PCR AND RESTRICTION PROFILES FROM PRIMER SET YLEXP06 F2/ YLEXP06 R1 (Trial II).

Using the PCR amplified product from the primer set PBF F1/YLEXP06 F1 as a DNA template for amplification, PCR was performed using the primer set YLEXP06 F2/YLEXP06 R1 to obtain a CP gene suitable for ligation into pKOV136 using the recognition sites for *Bgl*II and *Avr*II. Amplification of a fragment of ~800 bp was observed on an agarose gel stained with ethidium bromide as indicated in Figure 2.3.11 A and the expected RFLP profile was obtained when the PCR product was cleaved with *Bam*HI and *Pst*I in separate reactions as indicated in Figure 2.3.11 B. The expected RFLP profile for *Bam*HI was two fragments of 186 bp and 596 bp and that for *Pst*I was three fragments of 132 bp, 218 bp and 432 bp.

Figure 2.3.11: A 1% w/v agarose gel visualised under UV illumination showing the PCR products from the primer set YLEXP06 F2/YLEXP06 R1 using PCR products from amplification using primer set PBF F1/YLEXP06 F1 as DNA template [A]. Figure [B] indicates the RFLP profiles of PCR products that were cleaved separately with *Bam*HI and *Pst*I (lanes 1 & 3 for *Bam*HI and lanes 2 & 4 for *Pst*I), respectively. The expected profiles for *Bam*HI and *Pst*I were observed for both PCR products. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

Numerous attempts to ligate the PCR product into pGEM™ TEasy Vector I were unsuccessful. Small scale plasmid isolations cleaved with *Eco*RI either showed the absence of inserts or incorrect RFLP profiles. The PCR product was cleaved with *Eco*RI to confirm that it was the CP gene and the correct profile was observed indicating that the correct gene was being used in the ligations.

For successful ligation of a gene into pGEM™ TEasy Vector I, it is important for sufficient A-tailing to occur. The presence of the base at the 5'-end of the primer is a great determinant in whether A-tailing will occur at the 3'-end of the complimentary strand of the PCR product and therefore enable ligation into the multiple cloning site of pGEM™ TEasy Vector I. A 5'-G or 5'-C on the primer allows A-tailing to occur at a higher degree, respectively than a 5'-T which incorporates the A less efficiently or a 5'-A where the 3'-T gets deleted. It was then suggested that the reason for unsuccessful ligation into pGEM™ TEasy Vector I could be as a result of insufficient A-tailing during PCR amplification thereby preventing the ligation of the PCR product to the 3'-T overhangs present at the insertion site as the forward primer YLEXP06 F2 begins with the base A (5'- AGA TCT ATG TGG GGC ACC TCT AAC TGC GCA T - 3'). For this reason a new forward primer YLEXP07 F3 was designed which included a 5'-C just before the recognition site for *Bgl*I (A▼GATC▲T) in the primer sequence.

2.3.5. <u>PCR AND RESTRICTION PROFILES FROM PRIMER SET YLEXP07 F3/ YLEXP06 R1.</u>

PCR amplification using the primer set YLEXP07 F3/YLEXP06 R1 yielded a fragment of ~800 bp [Figure 2.3.12 A]. When the product was cleaved with *Bam*HI and *Eco*RI in separate reactions the expected RFLP profiles of 186 bp and 596 bp and 228 bp and 554 bp, respectively was observed as indicated in Figure 2.3.12 B.
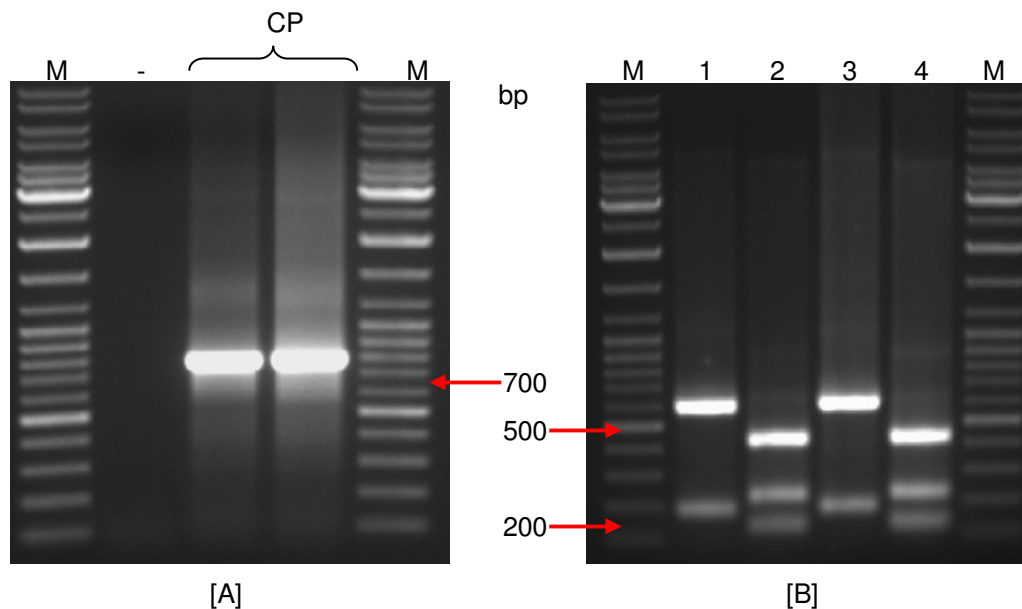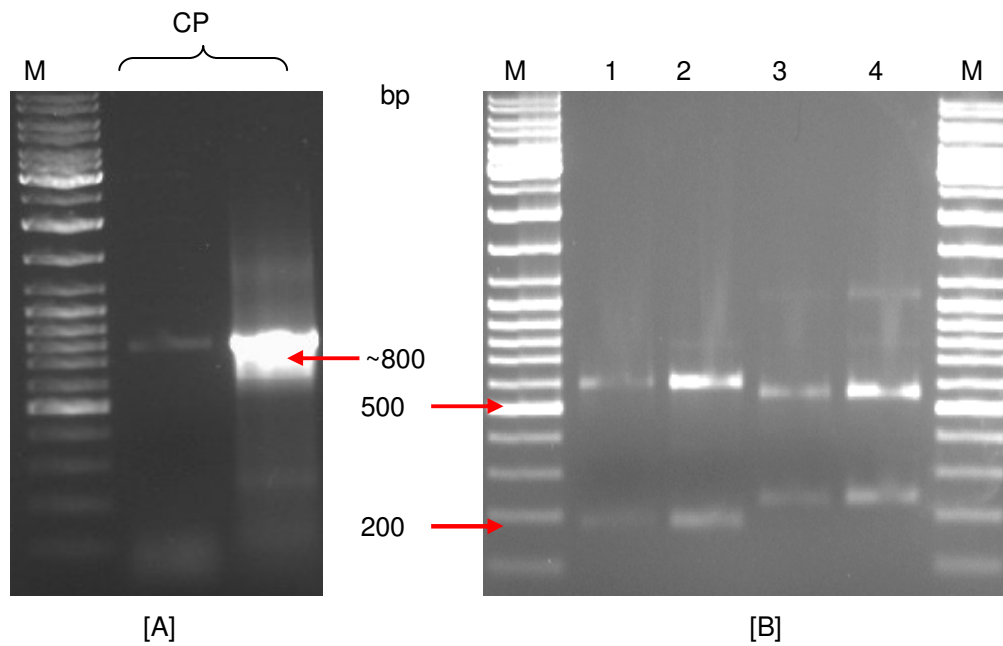
Figure 2.3.12: A 1% w/v agarose gel visualised under UV illumination showing the PCR products from the primer set YLEXP07 F3/YLEXP06 R1 [A]. Figure [B] indicates the RFLP profiles of PCR products that were cleaved separately with *Bam*HI and *Eco*RI (lanes 1 & 2 for *Eco*RI and lanes 3 & 4 for *Bam*HI), respectively. The expected profiles for *Eco*RI and *Bam*HI were observed for both PCR products. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

The PCR products were ligated into pGEM™ TEasy Vector I and small scale plasmid isolations confirmed for the presence of inserts using *Eco*RI. Expected *Eco*RI profiles of plasmids with inserts consisted of three fragments 228 bp, 554 bp and 3015 bp (pGEM™ TEasy vector backbone) as shown in Figure 2.3.13 A. Plasmids with inserts (isolates from lanes 2, 3, 6, 7, 9, 10 and 14 from Figure 2.3.13 A) were further confirmed to have *Bgl*II and *Avr*II recognition sites using a double digest with the two restriction endonucleases which simply removed the entire insert of 782 bp from the pGEM™ TEasy vector backbone [Figure 2.3.13 B].
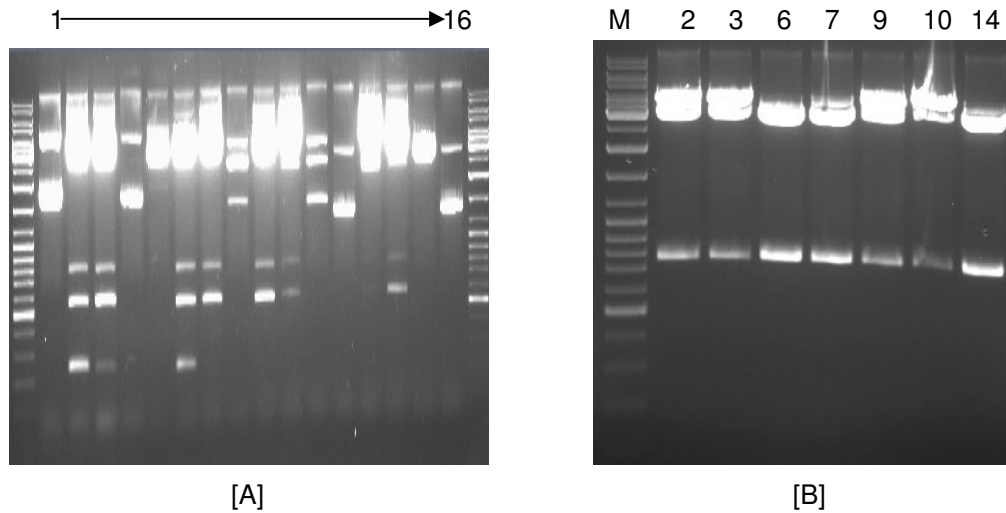
Figure 2.3.13: A 1% w/v agarose gel visualised under UV illumination confirming the presence of CP gene inserts in pGEM™ TEasy Vector using *Eco*RI [A] and *Bgl*II/*Avr*II [B].

Once the presence of inserts had been confirmed, plasmid isolations were cleaved with *Bgl*II/*Avr*II to remove the CP gene insert while pKOV136 was linearised with *Bam*HI/*Avr*II. As *Bgl*II and *Bam*HI have compatible sticky ends, ligation of the CP gene into pKOV136 was possible. Plasmid isolations were confirmed by a series of cleavages using *Pst*I, *Eco*RI and *Bsm*I/*Avr*II in separate reactions [Figure 2.3.14 A, B and C] and some isolates (clones 2, 6 and 14) were sent to Inqaba Biotechnologies Ltd for sequencing. Expected RFLP profiles for cleavage with *Pst*I, *Eco*RI and *Bsm*I/*Avr*II were observed for most of the plasmid isolations.
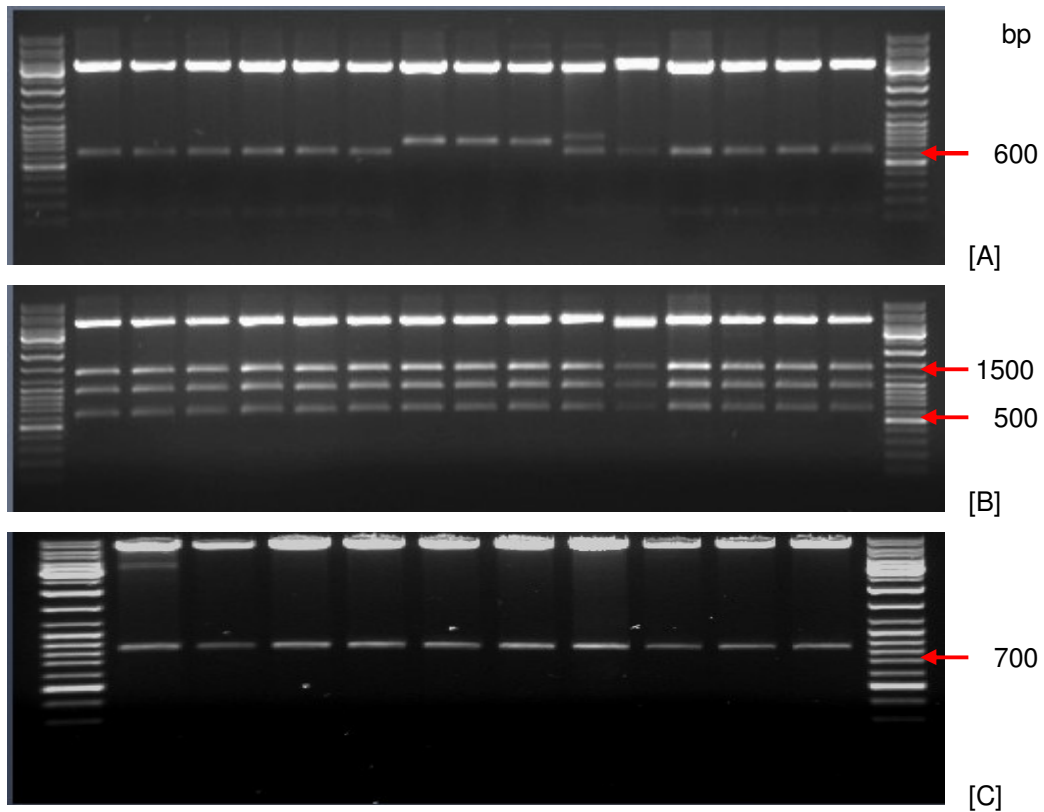
Figure 2.3.14: 1% agarose gels visualised under UV illumination confirming the presence of CP gene inserts in pKOV136 when cleaved with *Pst*I [A], *Eco*RI [B] and *Bsm*I/*Avr*II [C].

2.3.6. SEQUENCING RESULTS FOR COAT PROTEIN GENE FROM YLEXP07 F3/ YLEXP06 R1.

Sequences for three clones (2, 6 and 14) of isolated pKOV136/BFDV CP were obtained and aligned using DNassist v2.3. The sequences were not 100% similar as shown in Figure 2.3.15 with 3 base differences occurring at different sites. The significance of these differences were unknown but if there was indeed a C missing in the sequence for clone 14 then the 6X His tag would have been out of frame.

Figure 2.3.15: Sequence alignment of 3 clones of pKOV136/BFDV CP indicating the start and end (red arrows) of the CP gene; 3 base differences were observed.

2.3.7. <u>EUKARYOTIC (YEAST) EXPRESSION OF COAT PROTEIN FROM VECTOR pKOV136/BFDV CP.</u>

The three clones of vector pKOV136/BFDV CP that were sent for sequencing were linearised with the restriction endonuclease *Not*I to facilitate homologous recombination with the yeast genome and then transformed into *Y. lipolytica* Po1g competent cells. Po1g transformants were streaked out onto YNB N_{5000} plates and then transferred to YPD plates 48 hrs later as shown in Figure 2.3.16. The Po1g transformants were then transferred to liquid medium and grown overnight after which the cells were harvested and lysed. SDS-PAGE and Western blotting was performed on cell lysates to detect the presence of recombinant CP.

Figure 2.3.16: A YPD plate showing *Yarrowia lipolytica* Po1g transformants.

Integration of exogenous DNA into the *Y. lipolytica* genome occurs by homologous recombination into the flanking regions of pBR322 (pKOV136 is a pBR322 derivative) using *Not*I (flanked by the homologous pBR322 regions) to linearise the plasmid. The competent Po1g cells used in the present study are derived from the wild-type strain W29 by transformation of Po1f strains with a pBR322-*URA3* vector, forming the docking platform that is used for homologous recombination.

2.3.8. <u>SDS-PAGE AND WESTERN BLOTS OF EUKARYOTIC (YEAST) EXPRESSED RECOMBINANT COAT PROTEIN.</u>

SDS-PAGE of Po1g cells lysed with Y-PER® Yeast Protein Extraction Reagent (PIERCE) yielded inconclusive results as very faint smears were seen on the gel and

a Western blot of the same lysates did not yield any chemiluminescent bands (data not shown). From the SDS-PAGE gel it appeared that there was too little protein in the sample which could have been due to insufficient cell lysis and so the Po1g cells were subsequently lysed using glass beads. An SDS-PAGE gel of these lysates indicated more protein although it was difficult to conclusively indicate recombinantly expressed CP on the gel [Figure 2.3.17]. A Western blot, once again did not yield any protein bands (data not shown).
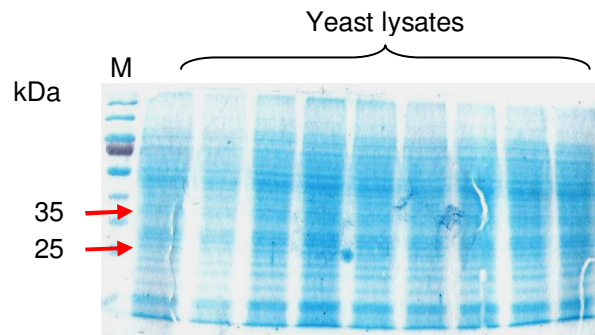
Figure 2.3.17: A 10% SDS-PAGE gel stained with PageBlue™ Protein Staining Solution separating proteins from Po1g cells lysed using glass beads. Lane M represents PageRuler™ Prestained Protein Ladder (Fermentas).

Additional concentration of the protein samples using TCA precipitation and separation on an SDS-PAGE gel once again yielded inconclusive results [Figure 2.3.18 A] and a Western blot revealed many bands using chemiluminescence, one of which was suspected to be recombinantly expressed CP [Figure 2.3.18 B]. Colorimetric detection of the lysates also indicated the possibility of recombinantly expressed CP [Figure 2.3.18 C] but none of these techniques were conclusive for detection of CP.
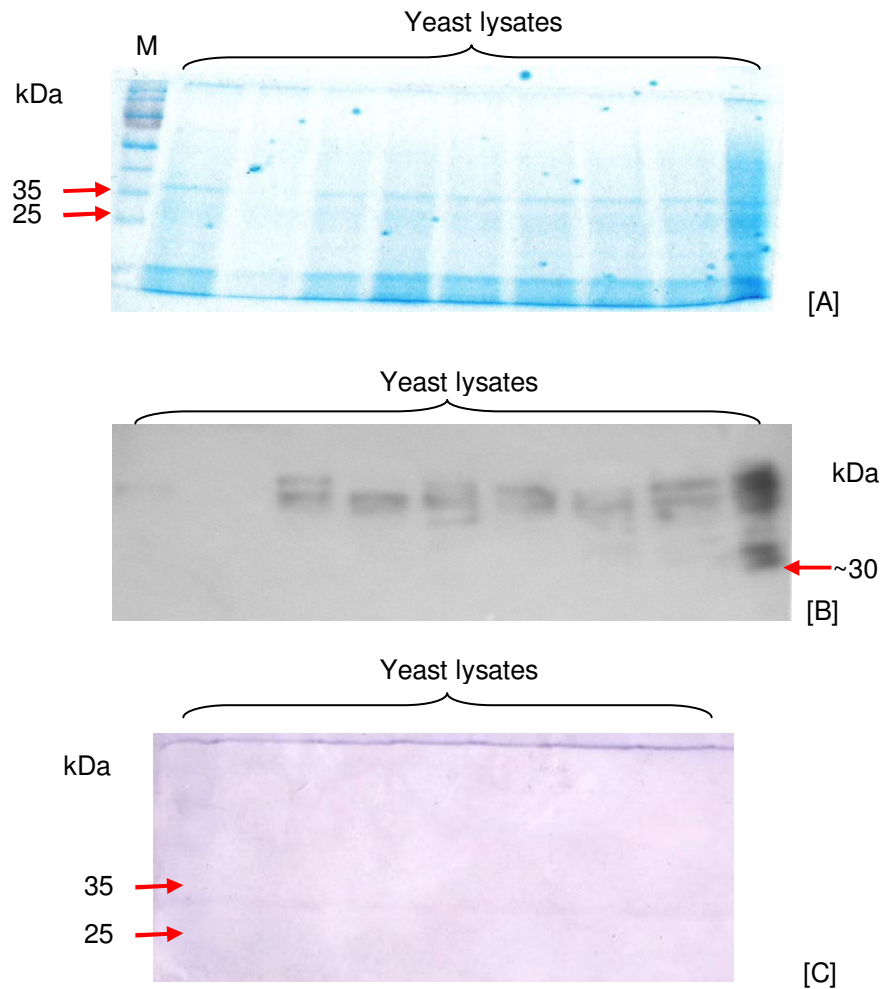
Figure 2.3.18: A 10% SDS-PAGE gel stained with PageBlue™ Protein Staining Solution separating TCA precipitated protein samples from Po1g cells [A] and a Western blot stained of the same samples stained chemiluminescently [B] and colorimetrically [C]. Lane M represents PageRuler™ Prestained Protein Ladder (Fermentas).

2.3.9. ELISA USING EUKARYOTIC (YEAST) EXPRESSED RECOMBINANT COAT PROTEIN.

The crude lysates from Po1g cells suspected to have expressed CP were used to coat an ELISA plate to determine if rabbit raised anti-BFDV would bind and detect the recombinantly expressed CP. No antigen-antibody reaction was observed in wells coated with yeast lysates while wells coated with known recombinant CP showed a positive yellow colour reaction reflective of antigen-antibody binging [Figure 2.3.19]. The positive antigen-antibody reaction obtained for wells coated with known recombinant CP indicated that the ELISA was functional while the lack of colour

development from wells coated with the yeast lysate indicated that antibodies had not bound to the antigen used to coat the plate. These results could be an indication of the complete lack of expression of recombinant CP from yeast or that the recombinant protein was present but in undetectable concentrations.
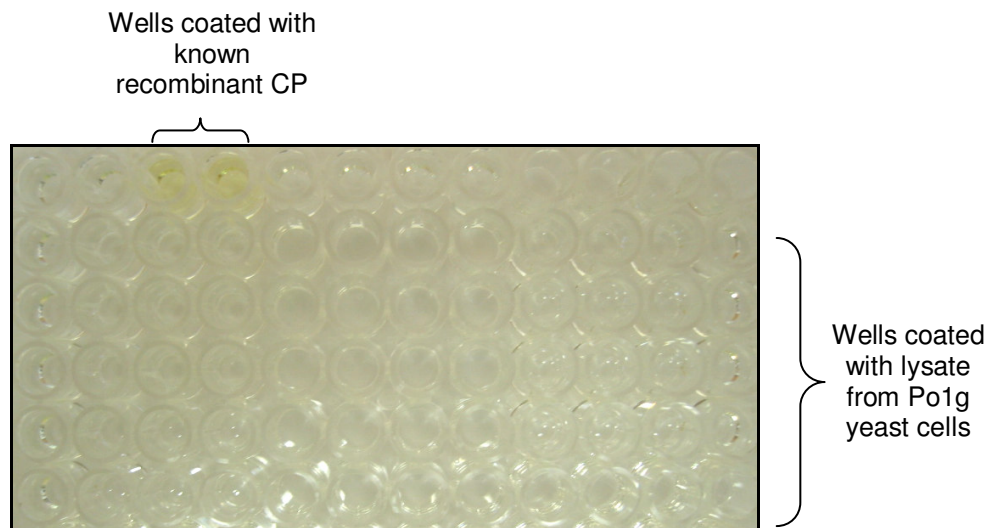
Figure 2.3.19: An ELISA plate coated with crude lysates from Po1g *Y. lipolytica* cells. No colour reaction was observed in these wells but a positive colour reaction was observed in two wells coated with known recombinant CP.

There are two major types of expression vectors described for *Y. lipolytica* expression systems: episomal replicative vectors and integrative vectors. pKOV136 is an integrative vector that allows the integration of exogenous DNA into the *Y. lipolytica* genome by homologous recombination to a docking platform. This is strongly stimulated by the linearization of the plasmid with the restriction endonuclease *Not*I. In more than 80% of the cases, a single copy of the vector will be integrated at the selected site offering several advantages that include very high transformation efficiency and precise targeting of the monocopy integration into the genome (Gellissen *et al.*, 2005). However, one of the reasons for the lack of detectable recombinant CP in the present study could be attributed to lack of expression of the protein from Po1g cells. Although pKOV136 uses the TEF (transcription elongation factor) promoter which is highly constitutive for control of expression and is one of two strong promoters derived from *Y. lipolytica TEF* and *RPS7* genes that is suitable for the isolation of new enzyme genes by expression cloning, it is not recommended for heterologous production per se (Gellissen *et al.*, 2005).

It is also probable that the concentration of expressed recombinant CP was too low for detection using chemiluminescence as well as using an ELISA [at least 1 µg.ml^{-1} of coating antigen is required for an ELISA to function optimally (Technical Guide for ELISA at www.kpl.com)]. Low expression levels may be attributed to single-copy expression; transformants have been reported to carry up to 60 integrated copies present as tandem repeats at one or two genomic sites but only around 10 copies have been found to be stable in cases of detrimental gene products (Gellissen *et al.*, 2005). Nicaud and co-workers (2002) observed an 88-fold increase in productivity of heterologous proteins between a single copy integrant (mono-copy pINA1267-*lap A* vector in Po1g) grown in batch and a multi-copy integrant (multi-copy pNFF331-*lap A*-His$_6$ vector in YLP21) grown in fed batch.

2.3. [B] RESULTS AND DISCUSSION FOR BACTERIAL EXPRESSION.

2.3.10. PCR AND RESTRICTION PROFILES FROM PRIMER SET ECEXP07 F1/ ECEXP07 R1.

PCR amplification of the DNA template using the primer set ECEXP07 F1 and ECEXP07 R1 yielded a fragment of ~800 bp as indicated in Figure 2.3.20 A and was confirmed by cleavage with the restriction endonucleases *Bam*HI and *Eco*RI in separate reactions [Figure 2.3.20 B]. The expected profiles for *Bam*HI and *Eco*RI were 169 bp and 594 bp and 219 bp and 544 bp, respectively. The PCR product was ligated to the vector pGEM™ TEasy and small scale plasmid isolations confirmed for the presence of inserts by cleavage with *Eco*RI [Figure 2.3.21, lanes 1-3 and lanes 5-9].
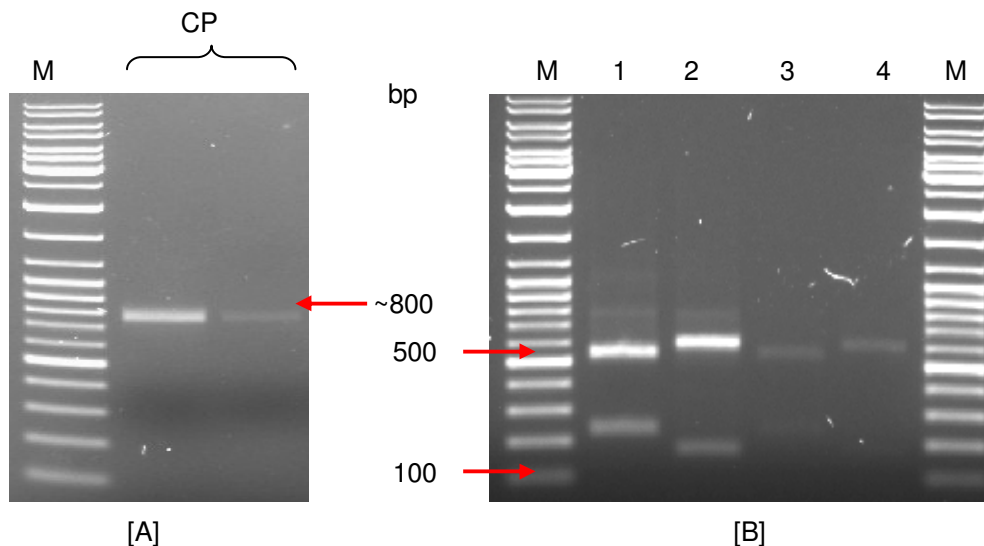
Figure 2.3.20: A 1% w/v agarose gel visualised under UV illumination showing the PCR products from the primer set ECEXP07 F1/ECEXP07 R1 [A]. Figure [B] indicates the RFLP profiles of PCR products that were cleaved separately with *Bam*HI and *Eco*RI (lanes 1 & 3 for *Eco*RI and lanes 2 & 4 for *Bam*HI), respectively. The expected profiles for *Eco*RI and *Bam*HI were observed for both PCR products. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.
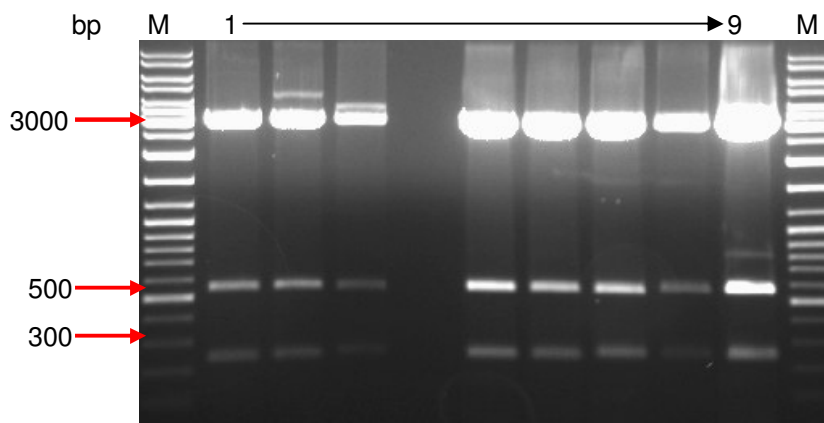
Figure 2.3.21: 1% agarose gel visualised under UV illumination confirming the presence of CP gene inserts in pGEM™ TEasy (clones 1-9) when cleaved with *Eco*RI. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

CP inserts were removed from pGEM™ TEasy Vector I using a double digest with the restriction endonucleases *Bgl*II/*Hind*III and the same set of endonucleases were used to linearise the vector pBAD/His B (Invitrogen). The CP gene was then ligated to the

vector pBAD/His B and transformed into competent TOP10 *E. coli* cells which are *rec*A, *end*A strains capable of transporting L-arabinose but not metabolizing it so that L-arabinose concentrations remain constant in the cells during expression studies and not decrease over time. Small scale plasmid isolations were confirmed to contain inserts by cleavage with *Bgl*II/*Hind*III [Figure 2.3.22 A] and plasmids containing inserts were checked for the correct orientation using *Bam*HI (isolates in lanes 1-10 in Figure 2.3.22 B all have inserts in the correct orientation). The expected RFLP profile for plasmids containing inserts in the correct orientation was 172 bp, 607 bp and 4037 bp.
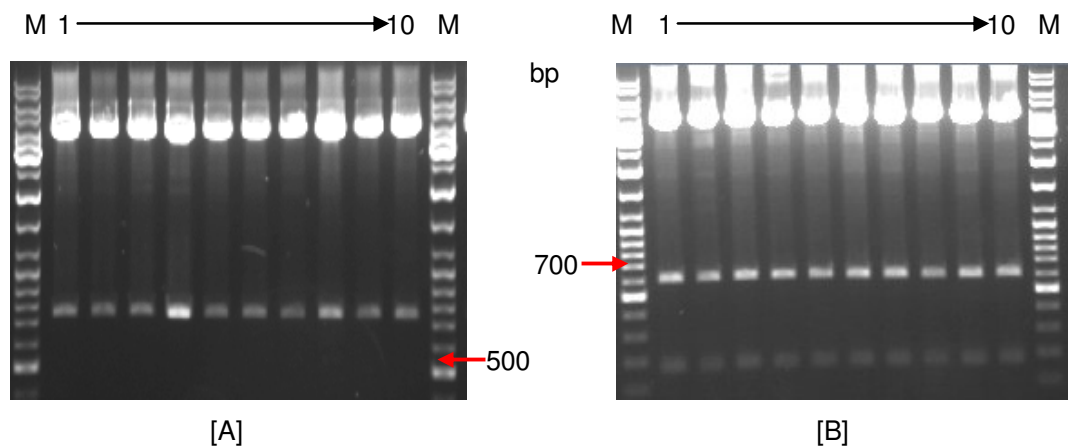
Figure 2.3.22: 1% agarose gels visualised under UV illumination confirming the presence of CP gene inserts in pBAD/ His B (clones 1-10) when cleaved with *Bgl*II/*Hind*III [A] and *Bam*HI [B]. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

Plasmids containing CP gene inserts in the correct orientation (pBAD/His/ BFDV CP) together with the control plasmids pBAD/His B and pBAD/His/*lac*Z (Invitrogen) were inoculated into LB broth containing ampicillin and small scale plasmid isolations confirmed using *Bam*HI and *Bgl*II/*Hind*III in separate reactions [Figure 2.3.23 A and B]. Positive isolates were then used to make glycerol stocks and subsequently used in all downstream reactions.

74
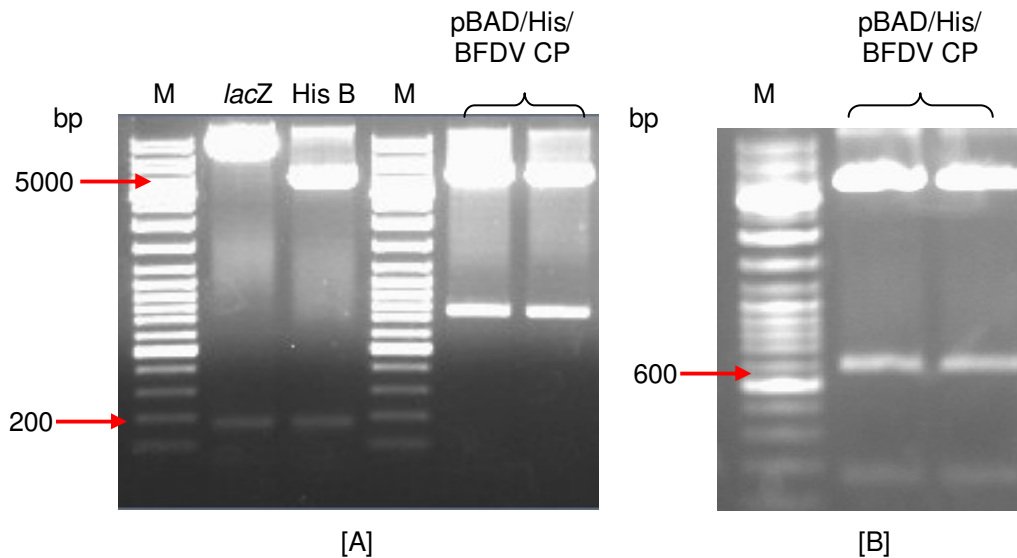
Figure 2.3.23: 1% agarose gels visualised under UV illumination showing linearised control plasmids pBAD/His/*lac*Z and pBAD/His B (lanes *lac*Z and His B, respectively) cleaved with *Bam*HI and confirming the presence of CP gene inserts in pBAD/His/BFDV CP when cleaved with *Bgl*II/*Hind*III [A] and *Bam*HI [B]. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

2.3.11. SDS-PAGE AND WESTERN BLOTS OF BACTERIALLY EXPRESSED RECOMBINANT COAT PROTEIN.

E. coli cells containing either pBAD/His B, pBAD/His/*lac*Z or pBAD/His/BFDV CP were grown to an OD_{600} of ~0.5 and induced with a final concentration of 0.02% and 0.002% L-arabinose. Pellet samples were collected at time t=0 hrs and t=4 hrs and run on SDS-PAGE gels. A Western blot showing protein bands of the expected sizes for ß-galactosidase (~120 kDa) and CP (~30 kDa) observed in the t=4 hrs samples with induction using 0.02% and 0.002% L-arabinose is shown in Figure 2.3.24. No bands were detected in the t=0 hrs samples.
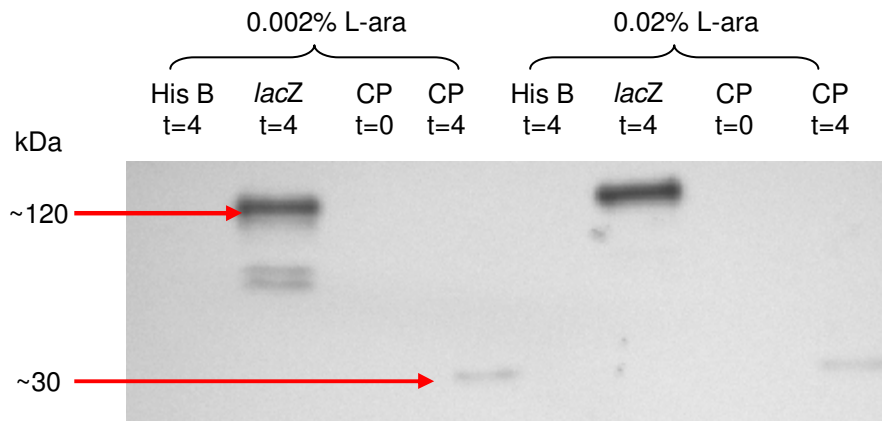
Figure 2.3.24: A Western blot showing the expression of recombinant ß-galactosidase (~120 kDa) from positive control plasmid pBAD/His/*lacZ* and recombinant CP (~30 kDa) from plasmid pBAD/His/BFDV CP 4 hours after induction with a final concentration of 0.002% and 0.02% L-arabinose.

When the expression period was extended for 8 hrs using a final concentration of 0.03% L-arabinose as inducer, the recombinant CP was found to be present in the insoluble fraction as opposed to the soluble fraction. This is indicated in Figure 2.3.25 where both ß-galactosidase and CP was detected in the insoluble fraction of samples collected 8 hrs post-induction and not in the soluble fraction.
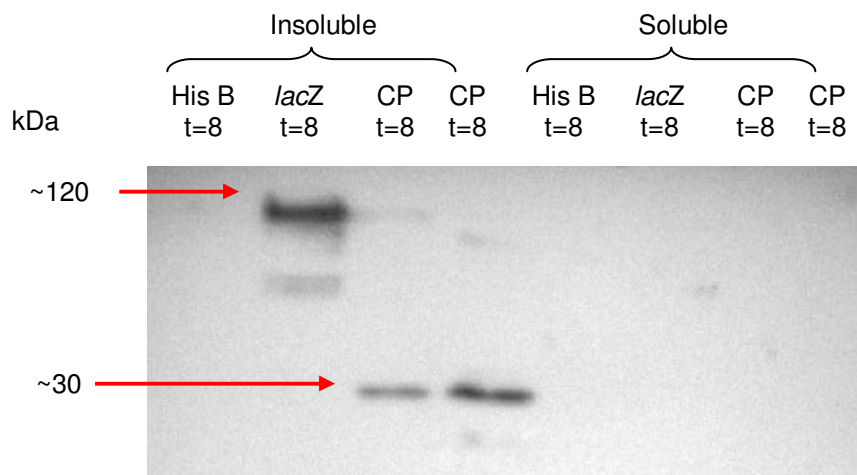
Figure 2.3.25: A Western blot showing the expression of recombinant ß-galactosidase (~120 kDa) from positive control plasmid pBAD/His/*lacZ* and recombinant CP (~30 kDa) from plasmid pBAD/His/BFDV CP 8 hours after induction with a final concentration of 0.03% L-arabinose. Recombinant protein was present only in the insoluble fraction.

Optimization studies carried out to determine conditions to be used for the expression of recombinant CP from bacterial cells was done using final concentrations of

0.002%, 0.02% and 0.2% of L-arabinose at temperatures of 25°C and 37°C. Samples were collected after 1 hr, 3 hrs and 6 hrs and both soluble and insoluble fractions were separated on 10% SDS-PAGE gels as indicated in Figures 2.3.26 A, B and C where the SDS-PAGE gels for *E. coli* cells induced with a final concentration of 0.2% L-arabinose are shown. At 25°C [Figure 2.3.26 A], more recombinant protein was observed in the insoluble fractions while at 37°C [Figure 2.3.26 B] higher protein concentrations were observed in the soluble fraction.
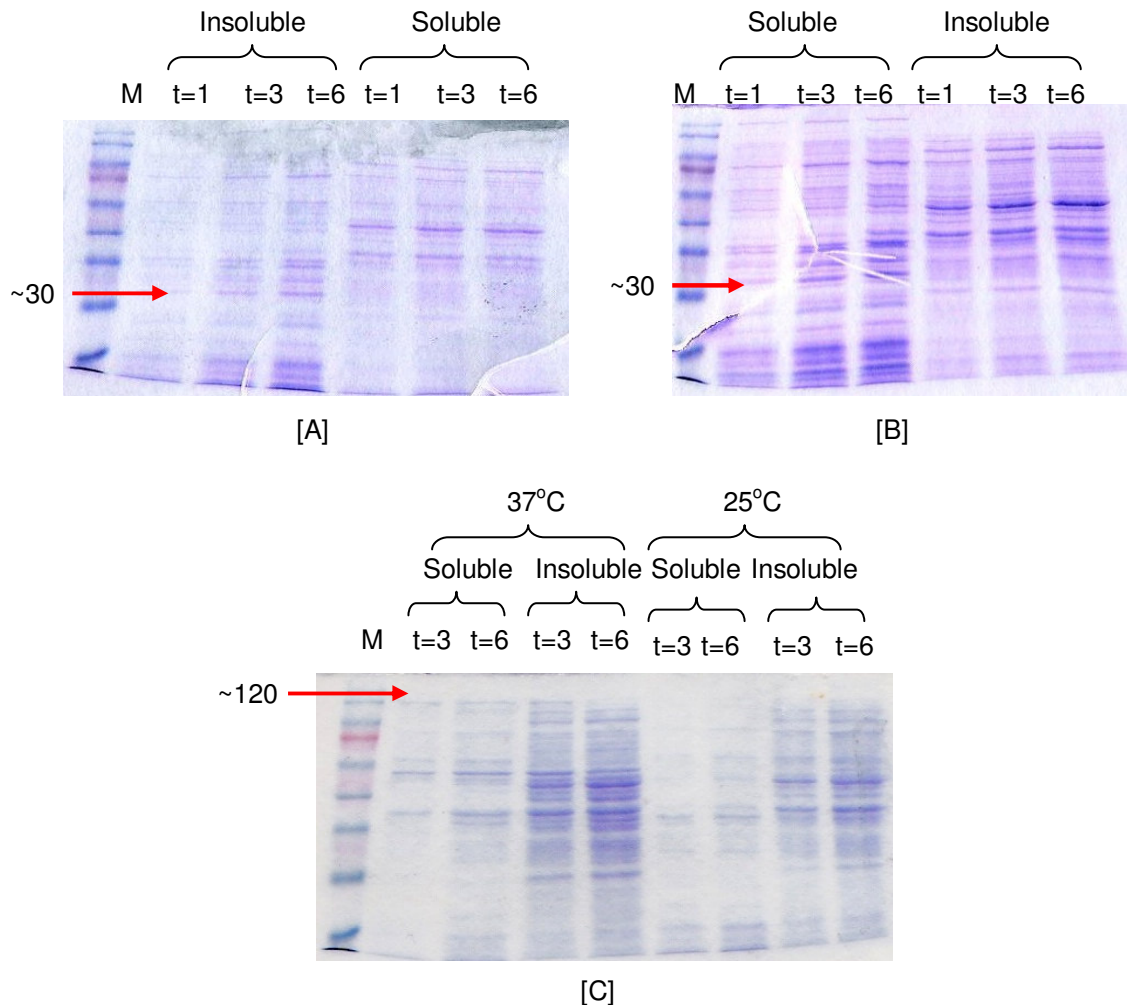
Figure 2.3.26: A 10% SDS-PAGE gel stained with Coomassie blue indicating the presence of recombinant CP either in the insoluble fraction of cells harvested 1, 3 and 6 hours after induction of *E. coli* cells with a final concentration of 0.2% L-arabinose grown at 25°C [A] or in the soluble fraction when the cells were induced with the same concentration of L-arabinose but grown at 37°C [B]. Figure [C] shows the presence of recombinant ß-galactosidase in the soluble fraction and insoluble fraction when *E. coli* cells were grown at 37°C and 25°C, respectively and induced with a final concentration of 0.2% L-arabinose. Lane M represents the PageRuler™ Prestained Protein Ladder (Fermentas).

The pBAD plasmid system is designed for regulated, dose-dependent recombinant protein expression and purification in *E. coli* allowing optimum levels of soluble recombinant protein to be produced using the *ara*BAD promoter (P_{BAD}) in *E. coli*. The AraC protein is both a positive and negative regulator and in the presence of L-arabinose, transcription from the P_{BAD} promoter is turned on while in its absence, transcription occurs at very low levels (Guzman *et al.*, 1995). By varying the concentration of L-arabinose, protein expression levels can be optimized to ensure maximum expression of soluble protein.

Optimum expression conditions obtained for the present study included growth at $37^{o}C$, induction with a final concentration of 0.2% L-arabinose and harvesting of cells 6 hrs post-induction and were used for subsequent CP expression. When the temperature was reduced to $25^{o}C$ or the harvesting time extended to 8 hrs, inclusion bodies containing the recombinant CP were formed as indicated by the presence of recombinant CP in the insoluble fractions in Figures 2.3.25 and 2.3.26 A. Over-expression in the cytoplasm is one of the major disadvantages of expression of heterologous proteins in *E. coli* because these proteins form inclusion bodies. Contrary to the strategies used for resolving the formation of inclusion bodies such as lowering temperature and lowering inducer concentration (Terpe, 2006), the present study indicated that recombinant CP was present in the soluble fraction at $37^{o}C$ as compared to $25^{o}C$ and when cells were induced with a final concentration of 0.2% L-arabinose as opposed to 0.002%, 0.02% and 0.03% L-arabinose.

The detrimental effects of overproduction of heterologous proteins like lethality and formation of inclusion bodies in numerous systems that use strong inducible promoters have been alleviated by replacing the vector in use with pBAD plasmids (Guzman *et al.*, 1995). The excellent modulation of the P_{BAD} promoter expression is fine-tuned by the concentration of L-arabinose where strains deleted for *ara* genes can reach maximal induction with as little as 0.001% L-arabinose or in strains that ferment, 1% is necessary for full induction (Terpe, 2006). This dependence on the concentration of L-arabinose for modulation of different heterologous proteins and bacterial strains can be attributed for the differences observed during expression of recombinant CP in the soluble and insoluble fractions. However, it is not surprising to obtain maximal expression using a final concentration of 0.2% L-arabinose (optimum final concentration of L-arabinose used in the present study) for induction as Guzman and co-workers (1995) reported maximal activity of expressed AP at this concentration for both *ara*[+] and *ara* strains.

2.3.12. PURIFICATION OF BACTERIALLY EXPRESSED RECOMBINANT COAT PROTEIN.

Cells harvested 6 hrs post-induction with a final concentration of 0.2% L-arabinose were sonicated in 100 mM MOPS buffer (pH 7.4) containing 0.5 M NaCl and 20 mM imidazole and the soluble fraction purified on a HisTrap FF column (GE Healthcare). A profile of the purification was obtained for two samples 10 and 17 [Figure 2.3.27]. From the profile, a slight peak was observed between fractions 5 (67.5 ml) and 13 (87.5 ml) and these fractions were suspected to contain recombinant CP.
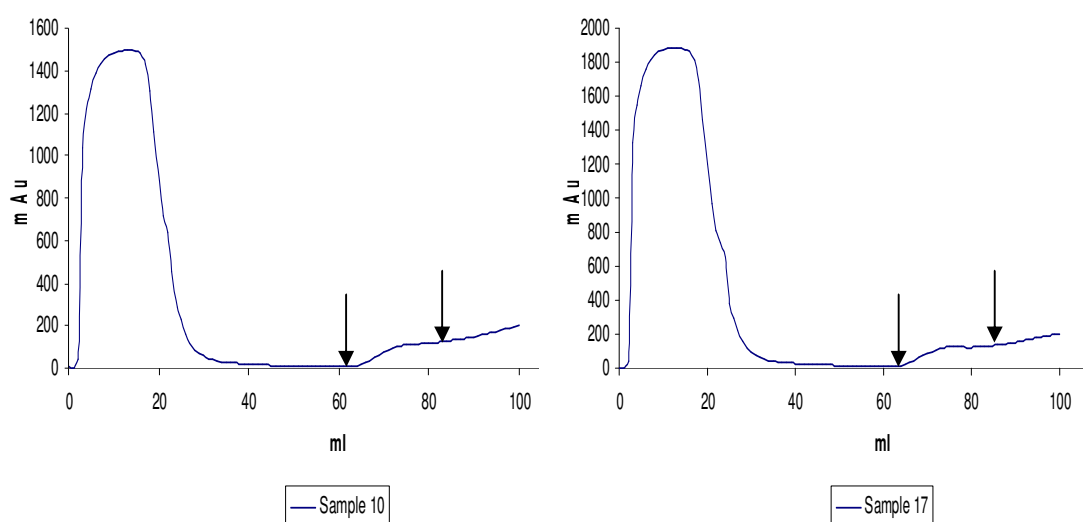
Figure 2.3.27: Purification profiles of two samples containing recombinant expressed CP from *E. coli* cells transformed with the vector pBAD/His/BFDV CP. Recombinant CP was purified on a HisTrap FF column and slight peaks were observed for fractions between 5 [67.5 ml] and 13 [87.5 ml] (indicated by the position of the arrows).

A sample from each fraction was separated on an SDS-PAGE gel as indicated in Figure 2.3.28. The concentration of recombinant CP (~30 kDa) increases in fractions from lanes 5 to lanes 9 and thereafter decreases for both samples. Recombinant CP was only partially purified as additional non-specific protein bands are visible in Figure 2.3.28. Over-sonication can lead to the co-purification of host proteins with the recombinant protein and may have been the reason why partial purification of the recombinant CP was achieved.
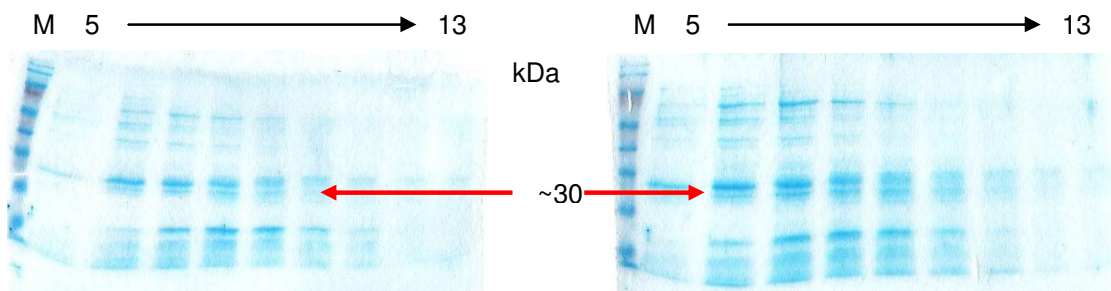
Figure 2.3.28: A 10% SDS-PAGE gel stained with PageBlue™ Protein Staining Solution separating proteins from fractions suspected to contain purified recombinant CP after purification of two samples through a HisTrap FF column. Lane M represents the PageRuler™ Prestained Protein Ladder (Fermentas).
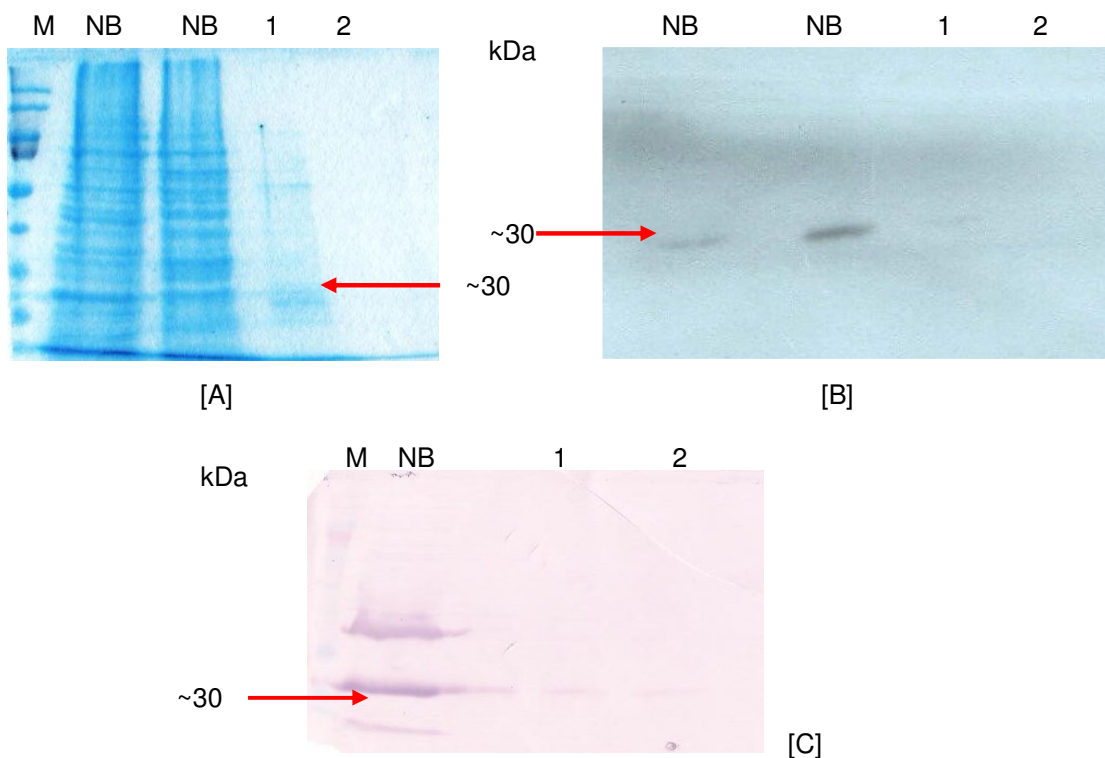
Figure 2.3.29: A 10% SDS-PAGE gel stained with PageBlue™ Protein Staining Solution showing the presence of recombinant CP in two non-binding fractions (Lanes NB) and in pooled fractions 1 and 2 of partially purified protein [A]. A Western blot of the same samples was detected chemiluminescently [B] while a second Western blot using only one non-binding fraction was detected colorimetrically using polyclonal rabbit-raised anti-BFDV [C]. The non-binding fractions contain higher concentrations of recombinant CP than the pooled partially purified fractions. Lane M represents the PageRuler™ Prestained Protein Ladder (Fermentas).

Fractions 5-12 were pooled and dialysed from each sample and separated on SDS-PAGE gels. An SDS-PAGE gel and Western blots of the pooled and dialysed fractions showed the presence of CP [Figure 2.3.29 A, B and C] when stained with PageBlue™ Protein Staining Solution (Fermentas) and for chemiluminescent and colorimetric detection, respectively, but these fractions showed very little protein when compared to non-binding fractions collected from the purification procedure. This indicates that a lot of recombinant CP was eluted and lost in the non-binding fraction.

The pooled fractions of one sample were re-run on the column to increase protein purity by increasing the imidazole concentration of the elution buffer from 20 mM to 40 mM and adding ß-mercaptoethanol. However, no apparent peak was observed in the purification profile as indicated in Figure 2.3.30 and no protein bands were observed for fractions 5, 7, 9, 10, 12, 14 and 17 when separated on a SDS-PAGE gel stained with PageBlue™ Protein Staining Solution (Fermentas) except in the non-binding fraction [Figure 2.3.31, lane NB]. A Western blot also showed no indication of the presence of protein.
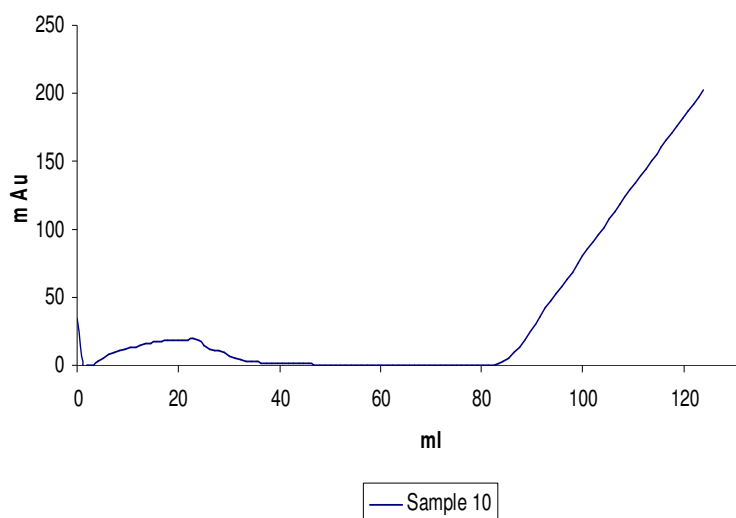
Figure 2.3.30: Purification profile of a pooled sample of partially purified recombinant CP reloaded on a HisTrap FF column. No apparent peak indicating the presence of purified recombinant CP fractions was observed.

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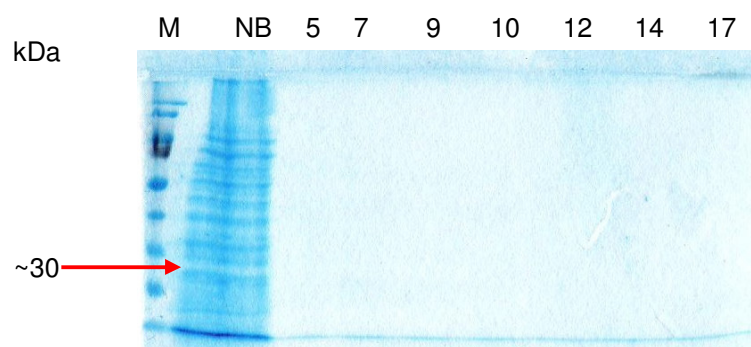
Figure 2.3.31: A 10% SDS-PAGE gel stained with PageBlue™ Protein Staining Solution showing presence of recombinant CP only in the non-binding fraction (Lane NB) of a re-purified sample on a HisTrap FF column. Lane M represents the PageRuler™ Prestained Protein Ladder (Fermentas).

Higher imidazole concentrations make the elution buffer more stringent removing impurities from the column and resulting in a higher purity of the recombinant protein in the elution buffer while ß-mercaptoethanol disrupts sulphide bonds preventing the co-elution of contaminating proteins. However, ß-mercaptoethanol also affects the binding properties of the recombinant proteins and could be attributed for the almost complete loss of recombinant CP (only a small concentration of protein was present in the non-binding fraction) during re-purification. Partial purification and very low concentrations of purified protein were also obtained using the B-PER® 6xHis Fusion Protein Purification Kit [PIERCE] [Figure 2.3.32]. Once again recombinant CP was being eluted and lost in the non-binding fractions.
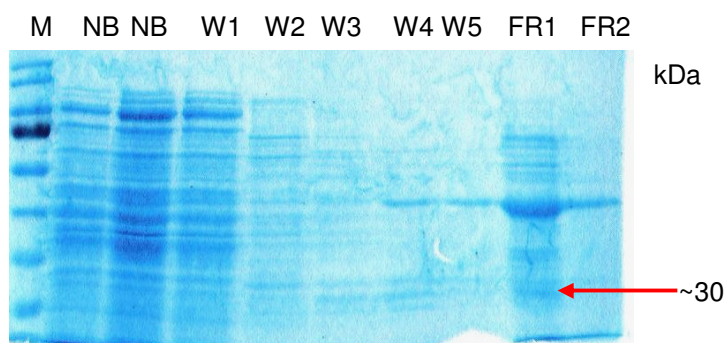
Figure 2.3.32: A 10% SDS-PAGE gel stained with PageBlue™ Protein Staining Solution showing the loss of recombinant CP in the non-binding fractions (Lane NB) and wash fractions (W1-W5) when purified using the B-PER® 6xHis Fusion Protein Purification Kit. Purified recombinant CP was present in low concentrations in the first fraction collected and absent in fraction 2 (Lanes FR1 and FR2, respectively). Lane M represents the PageRuler™ Prestained Protein Ladder (Fermentas).

2.3.13. <u>QUANTIFICATION OF PARTIALLY PURIFIED RECOMBINANT COAT PROTEIN USING BCA PROTEIN ASSAY.</u>

The total protein concentration of the pooled fractions of partially purified recombinant CP and that of rabbit raised anti-BFDV were obtained using the Micro BCA™ Protein Assay Reagent Kit (PIERCE). A standard curve of BSA standard versus its concentration was constructed [Figure 2.3.33] and the total protein concentration of each unknown sample determined.
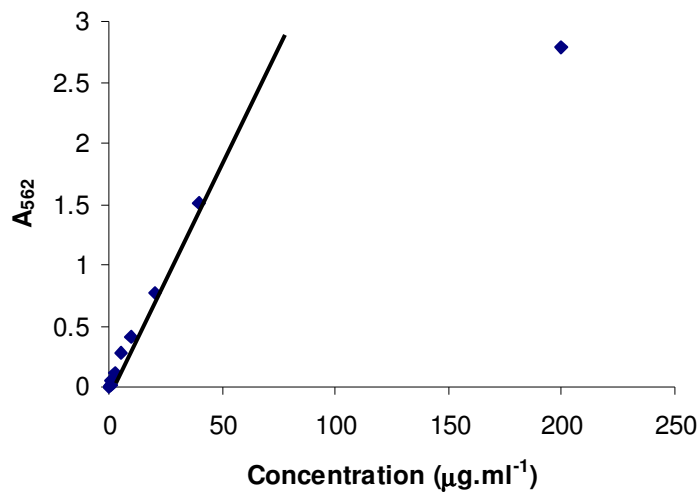
Figure 2.3.33: A BSA standard curve constructed from the A_{562} readings of known concentrations of BSA.

A total protein concentration of ~5 µg.ml^{-1} was obtained for a 1:10 dilution of the pooled CP fractions using the BSA standard curve. This means that the total protein concentration of the pooled fraction was ~50 µg.ml^{-1} for the undiluted sample.

2.3.14. <u>INDIRECT ELISA USING BACTERIALLY EXPRESSED RECOMBINANT COAT PROTEIN.</u>

Each well of the ELISA plate was coated with ~5 µg.ml^{-1} of recombinant CP extract while background controls were coated with 50 mM carbonate buffer (pH 9.6). When the concentration of the coating antigen was decreased to 2 µg.ml^{-1} the indirect ELISA assay became invalid as A_{450} readings became almost 0 (data not shown). This was due to the insufficient amount of recombinant CP available for antibody binding and detection. A typical protein coating solution of 1-20 µg.ml^{-1} can be used

as a starting point for successful plate coating when establishing ELISAs (Technical Guide for ELISA at www.kpl.com).

A yellow colour reaction in the ELISA indicated the binding of the antibody to the recombinant CP even though only partially purified recombinant CP was used as coating antigen [Figure 2.3.34 A]. The component to be coated onto the ELISA plate should be of high purity, preferably to homogeneity, which increases the sensitivity of the assay and reduces the potential for cross reactivity but for research purposes even crude lysate has been shown to function as antigen. The additional lack of antigen-antibody reaction in background controls (A_{450} readings obtained varied between 0.004 and 0.007, data not shown) where coating antigen (recombinant CP) was replaced with coating buffer (50 mM carbonate buffer, pH 9.6) also indicated the specificity of the binding of BFDV specific antibodies to recombinant CP.

The ELISA readings that were obtained when the indirect ELISA was carried out using rabbit-raised anti-BFDV, rabbit raised anti-BFDV adsorbed to *E. coli* cells and chicken raised anti-BFDV CP is represented in Figure 2.3.34 A and B. Positive antigen-antibody reactions (indicated by the development of a yellow colour) for polyclonal rabbit raised antibodies against BFDV and for antibodies that had been adsorbed to *E. coli* cells overnight were clearly observed up to a 10^{-3} dilution. From the graph, the highest absorbance reading for the rabbit raised anti-BFDV and adsorbed rabbit anti-BFDV was at the 10^{-2} dilution at 0.172 and 0.182, respectively while chicken raised anti-BFDV CP did not bind to bacterial expressed recombinant CP as indicated in Figure 2.3.34 A and B.

Figure 2.3.34: An ELISA plate indicating a positive antigen-antibody reaction when bacterially expressed partially purified recombinant CP used to coat the wells was reacted with rabbit raised anti-BFDV, rabbit raised Anti-BFDV adsorbed to *E. coli* cells and chicken raised anti-BFDV CP [A]. Figure [B] is a graph representing the absorbance readings at 450 nm versus the logarithm of the dilution factors used to dilute the antibodies tested.

The highest A_{450} reading obtained at a 10^{-2} dilution indicates the concentrations of recombinant CP and anti-BFDV required for optimal binding. This antigen-antibody reaction in the ELISA was indicated to be specific as similar A_{450} readings were obtained for rabbit anti-BFDV and adsorbed rabbit anti-BFDV antibodies. The chance of cross reactivity between *E. coli* antigens that may have been present in the partially purified recombinant CP sample and antibodies against *E. coli* that were present in the polyclonal antibodies was an important factor to consider when evaluating the ELISA. False-positive reactions may have resulted from this cross

reactivity since the recombinant CP was expressed in *E. coli* and rabbits are host to *E. coli*. If the development of a yellow colour after substrate was added was due to cross reactivity and not the specific antigen-antibody reaction then the rows that contained adsorbed anti-BFDV would not have resulted in a positive reaction as the antibodies would have been adsorbed to the *E. coli* cells and unavailable to bind to the coating antigen.

The positive indication of the use of bacterially expressed recombinant CP as coating antigen in an ELISA shown in the present study is comparable to the results that were obtained in a report by Johne *et al.* (2004). Johne and co-workers (2004) expressed a truncated form of recombinant CP in *E. coli* cells and used it as coating antigen in an ELISA to successfully detect BFDV specific antibodies in parrot sera. The parrot sera were detected using rabbit antibodies specific for IgG of an African grey parrot (*Psittacus erithacus*). The study reported three birds as being positive using HI assays, ELISA and immunoblot results and five birds as negative in all three tests. Two birds had negative HI and ELISA results but a faint band was detected by immunoblot while the last bird was regarded positive by HI and immunoblot but scored negative on the ELISA. Although the study reported that the results for the ELISA, HI and immunoblot correlated well, the test results of the three sera that did not correlate in all three tests could be due to the use of rabbit antibodies that were specific for IgG of an African grey parrot which may not have had cross reactivity with the individual birds. In a separate study by Shearer *et al.* (2008), full-length, baculovirus-expressed recombinant CP was successfully used in an indirect ELISA as coating antigen to screen mice injected with purified recombinant CP in Freund's incomplete adjuvant for the production of monoclonal antibodies against BFDV.

The indirect ELISA indicates that antibodies raised against whole virus can bind to recombinant CP which confirms that recombinant CP expressed from bacteria can be used as coating antigen in an indirect ELISA. These results also suggest that the opposite reaction is possible whereby antibodies raised against recombinant CP can bind to whole virus, lending support to the idea that a vaccine using recombinant CP as the antigenic determinant whether it is a sub-unit vaccine or a DNA vaccine encoding recombinant CP will result in protection of parrots infected naturally. However, it is also important to consider the presence of antigenic differences in different BFDV isolates when developing both a diagnostic ELISA using recombinant CP as well as a DNA or sub-unit vaccine. There may be a possibility that BFDV isolates are antigenically different as antibodies against purified recombinant CP that

were raised in chickens and detected using anti-chicken-HRP did not result in a positive antigen-antibody reaction in the same ELISA developed in the present study. This is an interesting observation as the recombinant CP (which was kindly donated to us by colleagues at UCT) was expressed from a different BFDV isolate to the isolate used in the present study. The lack of antigen-antibody binding may be due to distinctly different epitopic regions present on the CP used as coating antigen that the antibodies raised at UCT could not recognise inferring that the two BFDV isolates may be antigenically different. The only other suggestion of antigenic differences present in BFDV is in a report by Shearer *et al.* (2008) where sera from rainbow lorikeets (*Trichoglossus haematodus*), short-billed corellas (*Cacatua sanguinea*), a sulphur-crested cockatoo (*Cacatua galerita*), a red lorikeet (*Eos bornea*) and a galah-corella hybrid failed to inhibit agglutination from virus eluted from the feather of a cockatiel (*Nymphicus hollandicus*) suggesting that the cockatiel isolate may be sufficiently different antigenically to be considered a separate serotype.

2.3.15. <u>USE OF ANTI-CHICKEN ANTIBODIES TO DETECT PARROT ANTIBODIES.</u>

There are no commercially available anti-parrot conjugated antibodies to detect parrot raised antibodies directly in an ELISA. A test to verify the use of anti-chicken conjugated antibodies as a suggested alternative method to detect parrot antibodies in an indirect ELISA involved inoculating parrots with the La Sota NDV vaccine and detecting the parrot raised antibodies using a commercial FlockChek Newcastle Disease Virus Antibody Test Kit (Idexx) as well as by standard HA and HI assays. The ELISA results were correlated to the corresponding HI results as shown in Figure 2.3.35 A. Using results obtained from the manufacturer of the ELISA kit for testing chicken sera by both ELISA and HI assays when the FlockChek Newcastle Disease Virus Antibody Test Kit was being validated for diagnostic sensitivity a similar correlation graph was obtained as shown in Figure 2.3.35 B.
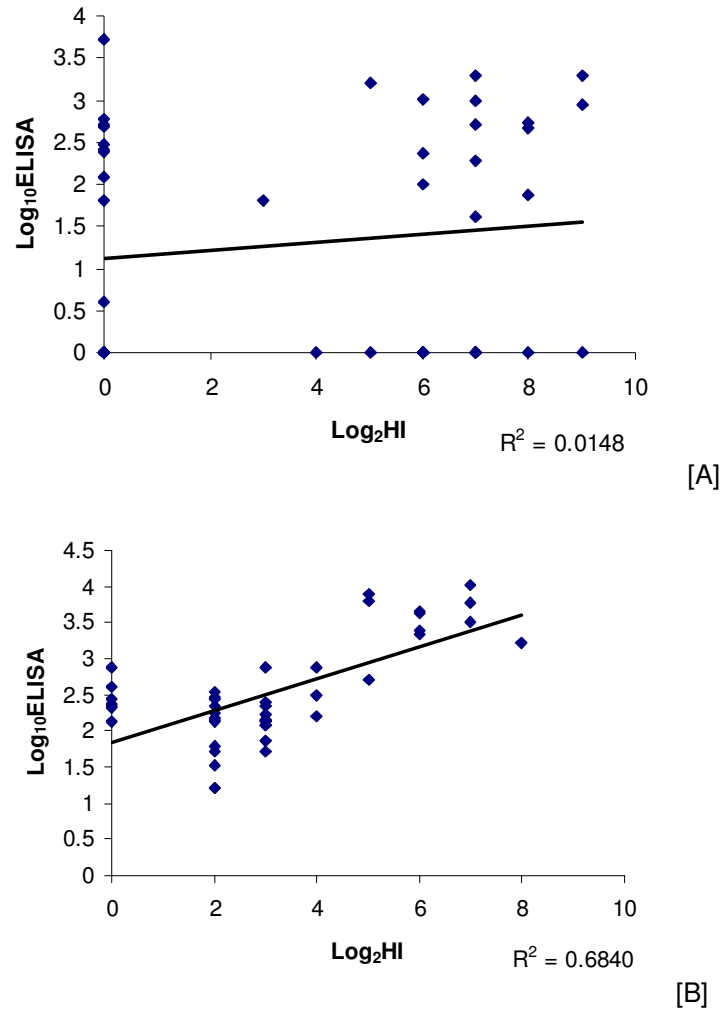
Figure 2.3.35: Correlation between ELISA and HI titres for parrots [A] and chickens [B] infected with NDV and detected using anti-chicken conjugated antibodies. The co-efficient of linearity is very low for the parrot sera (R^2=0.0148) while it is significant for chicken sera (R^2=0.6840).

From the graph correlating ELISA and HI titres from parrot sera, an insignificant correlation can be seen by the very low co-efficient of linearity value (R^2=0.0148). This indicates that the anti-chicken conjugated antibodies do not efficiently detect the parrot raised antibodies and so cannot be used in a direct ELISA to detect parrot raised anti-BFDV.

It is impractical, especially in South Africa, to use the concept of an indirect ELISA for the detection of BFDV specific antibodies in parrots. This is because there are no commercially conjugated secondary anti-parrot antibodies available for the detection of parrot antibodies. Although it is not difficult to make conjugated antibodies in the

laboratory, an important aspect to consider is cross reactivity. There are over 40 species of psittacines and there is no information available to indicate if there is cross reactivity between all these species so that, if for example antibodies are made against African grey parrots (*Psittacus erithacus*) it will be able to detect antibodies raised in sulphur-crested cockatoos (*C. galerita*) or in Galahs (*E. roseicapillus*). It is therefore necessary to establish another way of detecting parrot raised anti-BFDV.

Although there are no official reports confirming the use of anti-chicken antibodies to detect parrot raised antibodies, these antibodies are currently being used for this purpose (personal communication from Professor R. R. Bragg). However, as confirmed by the results obtained in the present study there is no significant relationship between titres of parrot serum containing antibodies against NDV when tested using a commercially available ELISA kit used to test chicken sera and the HI assays. Although positive reactions were seen when parrot serum was tested using the ELISA, the low co-efficient of linearity (R^2=0.0148) implies that the correlation between ELISA and HI titres is insignificant and consequently, anti-chicken can not be used to accurately detect and quantify parrot raised antibodies.

The alternative was to detect the parrot raised anti-BFDV in an indirect competitive ELISA using bacterial expressed recombinant CP as antigen and rabbit raised anti-BFDV as competing antibody.

2.3.16. INDIRECT COMPETITIVE ELISA USING BACTERIALLY EXPRESSED RECOMBINANT COAT PROTEIN.

Using a dilution of 10^{-2} for the rabbit raised anti-BFDV, an indirect competitive ELISA was performed to test the presence of parrot raised anti-BFDV in parrot sera suspected to be positive for PBFD. The results for the sera of five Cape parrots (*Poicephalus robustus*), an African grey parrot (*Psittacus erithacus*) and a Jardine (*Poicephalus gulielmi massaicus*) [previously tested as positive for PBFD using PCR testing at an independent molecular diagnostic laboratory] with the indirect competitive ELISA are shown in Figure 2.3.36 A and B.

Figure 2.3.36: Graphs of A_{450} versus log of the dilution factors used to dilute the parrot sera. A standard dilution of 10^{-2} of competing rabbit raised anti-BFDV was added to each well.
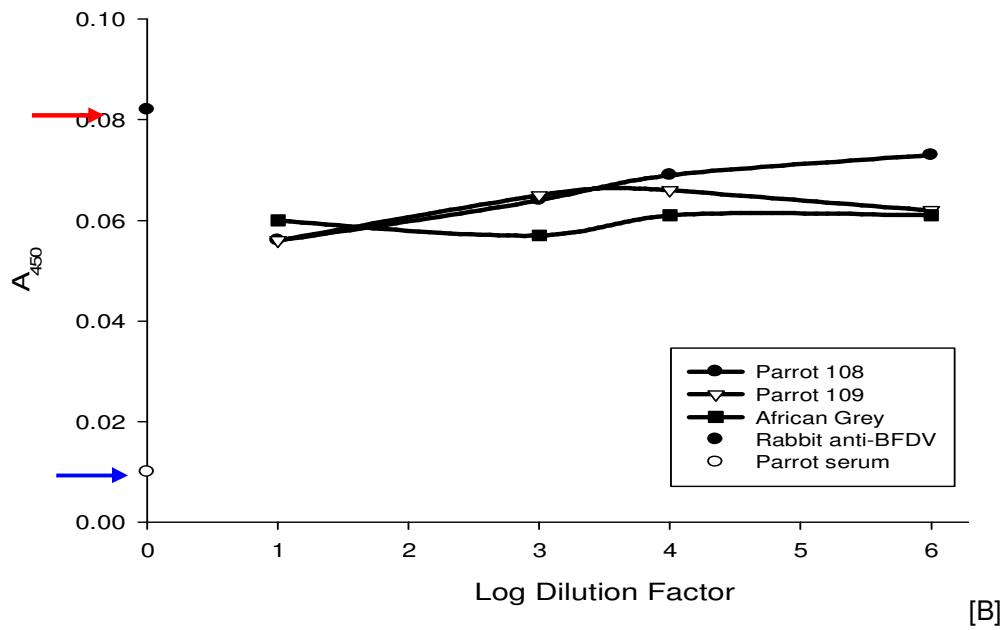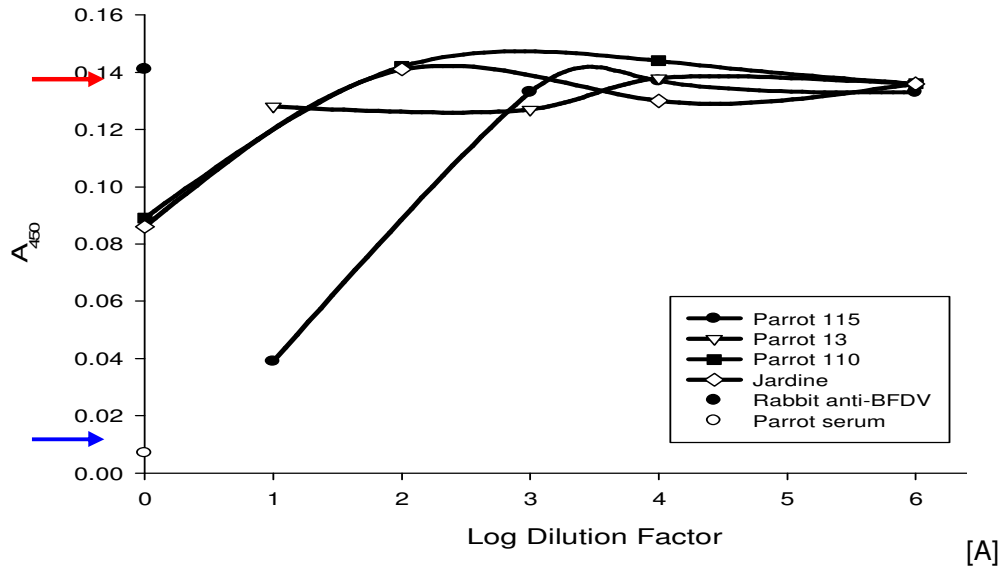
Dilution	Absorbance (450 nm)						
	A				B		
	Parrot 115	Parrot 13	Parrot 110	Jardine	Parrot 108	Parrot 109	African Grey
10^0			0.089	0.086			
10^{-1}	0.039	0.128			0.056	0.056	0.06
10^{-2}			0.142	0.141			
10^{-3}	0.133	0.127			0.064	0.065	0.057
10^{-4}	0.137	0.138	0.144	0.13	0.069	0.066	0.061
10^{-6}	0.133	0.136	0.136	0.136	0.073	0.062	0.061
	Rabbit anti-BFDV = 0.141				Rabbit anti-BFDV = 0.082		
	Parrot = 0.007				Parrot = 0.01		

The upper arrow indicates the point that represents the average titre observed when rabbit raised anti-BFDV was used without the addition of any competing antibodies of parrot origin and represents the highest possible titre which would be expected in birds without any antibodies against BFDV. The lower arrow indicates the point that represents a negative control point in which parrot serum was added to the wells without the addition of any rabbit raised antibodies. In this experiment the enzyme was conjugated to anti-rabbit and as such, no reaction was expected in any of these wells. From Figure 2.3.36 it can be implied that sera from Cape parrots 110, 115 and Jardine were all positive for antibodies against BFDV while that from Cape parrot 13 is negative for antibodies against BFDV. These results are indicated by the decrease in concentration of parrot antibodies as the dilution factor increases hence the increase in A_{450} readings. It appears that the sera from Cape parrots 108 and 109 and the African grey are negative as there is no obvious indication of the dilution of parrot raised antibodies as the dilution factor increases.

In a competitive ELISA, the signal obtained is inversely proportional to the concentration of analyte therefore the indirect competitive ELISA developed in the present study was based on the theory that the more parrot raised antibodies against BFDV there is, the more these antibodies will compete for binding sites on the coating antigen resulting in less rabbit raised antibodies being detected. This means that the higher the concentration of parrot raised anti-BFDV, the lower the absorbance reading due to less colour development while the lower the concentration of the parrot raised anti-BFDV the higher the absorbance readings at 450 nm. Any parrot serum that is negative for BFDV specific antibodies will have an A_{450} reading that is close to or equal to that of rabbit anti-BFDV without competing parrot serum. Using these guidelines, sera from Cape parrots 110 and 115 and Jardine that were tested using PCR and found to be positive were also positive for BFDV specific antibodies while Cape parrots 13, 108 and 109 and African grey all showed results that were negative for BFDV specific antibodies although they were PCR positive.

The partially purified recombinant CP was a limiting factor in the development of the ELISAs in the present study. As a possible alternative, non-binding fractions containing higher concentrations of recombinant CP (indicated during the purification procedure) were used in an indirect and competitive ELISA as coating antigen and where FBS was used as negative control serum. A number of additional controls were put in place to eliminate the possibility of background noise and these included wells with FBS minus primary antibody but including secondary antibody and wells

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that were not coated with recombinant CP and had FBS, primary and secondary antibody added to them. Two pools of rabbit raised anti-BFDV were tested and three pools of non-purified recombinant CP were used to coat the wells.

Little/no background reactions were observed indicating that the antigen-antibody reaction was specific and that non-purified recombinant CP can be used in an ELISA for research purposes. The indirect competitive ELISA was repeated with non-purified recombinant CP as coating antigen to test the "positive" parrot sera that was received and tested with the indirect competitive ELISA using partially purified recombinant CP in this study [Figure 2.3.37].

Serum Sample	A_{450}
FBS	0.108
108	0.088
109	0.099
110	0.114
African Grey	0.11
Rabbit anti-BFDV	0.12
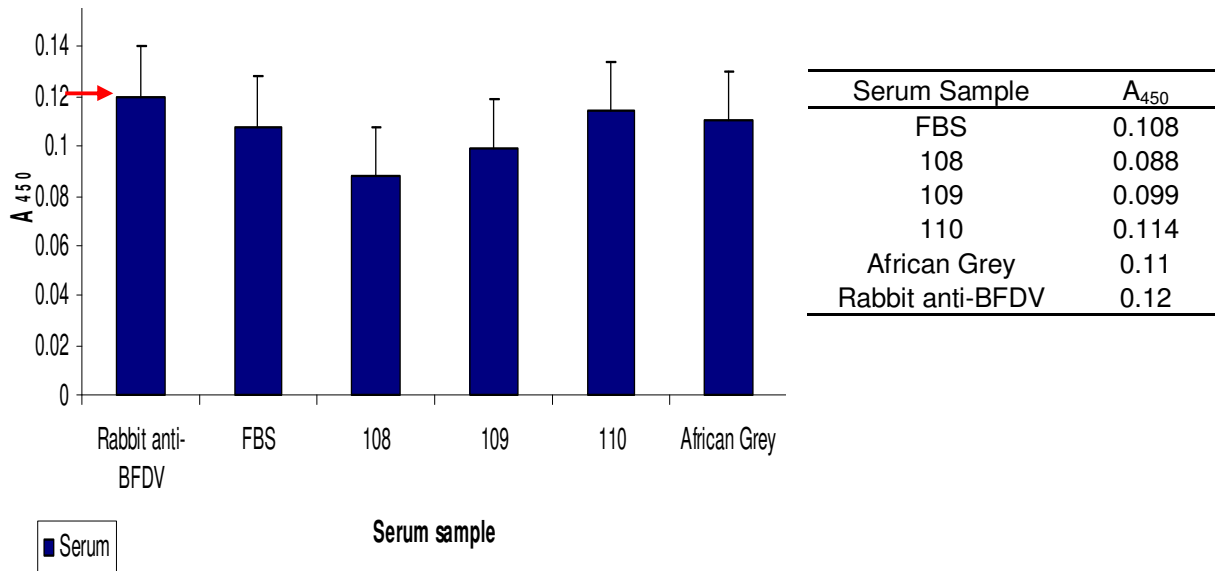
Figure 2.3.37: Graph of absorbance readings at 450 nm for FBS as a negative control and different parrot sera in comparison to the absorbance reading for rabbit anti-BFDV. The signal detected is inversely proportional to the concentration of parrot raised anti-BFDV.

FBS had a similar A_{450} reading to the rabbit anti-BFDV; parrot sera from the Cape parrot 110 and African grey also had similar A_{450} readings to rabbit anti-BFDV implying that these sera were negative for the presence of antibodies to BFDV. Serum from Cape parrots 108 and 109 had lower absorbance readings and could possibly be interpreted as being positive for anti-BFDV as the signal detected is inversely proportional to the concentration of parrot raised antibodies. These results differed from those obtained when the same sera were tested in the competitive

ELISA using partially purified recombinant CP where Cape parrot 110 was positive and Cape parrots 108 and 109 were negative.

These differences were due to the different dilutions used to test the sera. In the competitive ELISA using partially purified recombinant CP as coating antigen [Figure 2.3.36], the initial dilution of parrot sera tested for parrots 108, 109 and African grey was 1:10 while the serum for parrot 110 was undiluted. These ELISAs were done on different plates and the initial dilution was based on the volume of serum available for multiple tests. In the indirect competitive ELISA using non-purified recombinant CP as coating antigen [Figure 2.3.37], the sera were diluted 1:5. Using the A_{450} reading obtained for undiluted serum of parrot 110 (0.089) as a reference, a 5-fold dilution of this serum would dilute out the parrot anti-BFDV and bring the A_{450} reading closer to that of rabbit serum without added competing parrot serum hence, the negative reading in the competitive ELISA using non-purified recombinant CP. The same reason can be applied to sera from parrots 108 and 109 where at a 1:10 dilution, the parrot raised anti-BFDV was already diluted out and the ELISA results came up as negative. The reason for the dilution of the serum samples was due to very small volumes of serum which were available from the different birds. These results then indicate that Cape parrots 108, 109, 110 and 115 as well as Jardine were positive for BFDV specific antibodies. However, due to the inability to directly interpret the results obtained in this study as a standard titre used to classify serum as negative for anti-BFDV has not been established for the assay, these results can not be considered conclusive.

The detection of parrot raised anti-BFDV using the indirect competitive ELISA implies that both partially purified as well as non-purified recombinant CP can be used as coating antigen in ELISAs. Although, the reaction is specific for BFDV antibodies as confirmed by the various controls using FBS as negative serum as well as leaving wells uncoated with recombinant CP, parrot raised anti-BFDV that was successfully detected using the competitive ELISA can not be conclusively defined as protective or not. This is due to the lack of a cut-off value that accurately differentiates between antibody titres for BFDV positive birds and those that are negative; hence, the indirect competitive ELISA established here can not be used as a diagnostic ELISA.

To establish an ELISA as a diagnostic test it is important to standardize it and determine a cut-off value so as to validate results obtained. Without a cut-off value no conclusions can be made regarding the status of the source of serum. The

concentration dependence of competitive ELISAs requires that samples, or dilutions of initial samples fall within the relatively narrow concentration range of the most reliable portion of the standard curve and to construct a statistically accurate standard curve it is necessary to test a large number of samples that are known strong positive, weakly positive and negative sera. Muhamuda *et al.* (2007) standardized a competitive ELISA used to detect rabies neutralizing antibodies in vaccinated humans by using 50 samples each of known high positive, low positive and negative sera to determine the cut-off value (%) for ascertaining the level of inhibition. A study by Balamurugan and co-workers (2007) to develop an indirect ELISA for the detection of antibodies against Peste-des-petits-ruminants Virus in small ruminants also used large sampling numbers of known positive (n = 187) and negative (n = 382) serum samples to optimize the ELISA.

There are two limiting factors that restrain the standardisation of the indirect competitive ELISA established in the present study. Although between 10-20% of breeding stocks are lost annually to PBFD in South Africa, it is currently very difficult to obtain serum samples of infected birds from bird breeders as many prefer not to be public about any PBFD outbreaks in their aviaries. In addition to this, it is important for the samples to be accurately diagnosed as positive, weakly positive and negative in order to determine the cut-off value. Currently, birds are diagnosed mainly by PCR tests but these tests have been reported to be unreliable in asymptomatic birds (Kondiah, 2004) and so there is no diagnostic test available that is 100% reliable for detection of BFDV infection. It was therefore not possible, within the scope of this project, to standardize the indirect competitive ELISA and establish the percentage cut-off value to accurately distinguish between positive serum that is protective in parrots and negative serum. This aspect can be investigated in a separate study.

While the indirect competitive ELISA established in the present study can not be used as a diagnostic test, it has been used to successfully detect antibody responses in parrots that were naturally infected as well as in parrots that were vaccinated with a DNA vaccine encoding the CP gene and a sub-unit vaccine consisting of purified recombinant CP expressed in baculovirus (Chapter 3). If a large number of samples can be acquired that have been accurately diagnosed as positive or negative and the recombinant CP is purified to homogeneity, then it is possible to use the indirect competitive ELISA as a reliable diagnostic test for both symptomatic and asymptomatic birds.

2.4. CONCLUSIONS.

The need to find a different source of antigenic protein that will not restrain the diagnosis of PBFD as well as the development of reliable diagnostic tests and vaccines can be satisfied using recombinant expression systems that can be bacterial, eukaryotic, mammalian or insect cell based. Recombinant BFDV CP was successfully expressed in the present study using the pBAD bacterial expression system as opposed to the yeast expression system. Although only partial purification of the recombinant CP expressed in *E. coli* was achieved, the study has shown that both partially purified and non-purified recombinant CP can be used in indirect and indirect competitive ELISAs as coating antigen. The indirect competitive ELISA successfully detected BFDV specific antibodies in naturally infected and vaccinated parrots and can be established as a diagnostic test once it has been standardized. One of the most limiting factors in the development of reliable serological tests and a preventative vaccine for PBFD is the availability of wild-type virus but with the accessibility to a functional expression system as a source of antigenic protein, such limitations no longer pose as an obstacle into scientific research.

CHAPTER 3: DEVELOPMENT OF A DNA VACCINE CANDIDATE FOR PSITTACINE BEAK AND FEATHER DISEASE.

3.1. INTRODUCTION.

BFDV has a circular ambisense genome carrying seven ORFs (Bassami *et al.*, 2001) of which ORF 1 encodes the Rep protein while ORF 2 encodes the CP which has been suggested to be the epitopic protein of the virus. The Rep gene of the BFDV genome has been said to be the most conserved among all isolates while the CP gene has been reported to be genetically diverse in a number of studies. This genetic diversity has been associated with species specificity in a report by Ritchie *et al.* (2003) where three distinct lineages: a cockatoo lineage (CT), budgerigar lineage (BG) and a lorikeet lineage (LK) were identified while in another report by Bassami *et al.* (2001) genetic diversity has not been associated with regional differences or association with specific species. Heath and co-workers (2004) also discussed the existence of different genotypes, with three distinctly unique genotypes present in southern Africa, but did not find evidence to support species specificity. Reports by Raue *et al.* (2004) also support the existence of possible genotypes with no evidence of the species specificity observed by Ritchie and co-workers (2003) but de Kloet and de Kloet (2004) found a complex psittacine host specificity relationship that suggests that the relationship between BFDV isolates, psittacine species and pathogenicity is very complex.

Although there is a high degree of genetic diversity in the CP gene, there has been no report confirming the occurrence of antigenic differences. It is important to investigate the antigenic diversity that is most probably present in BFDV so as to develop a vaccine that will effectively protect parrots against all strains of this virus. However, for the purpose of this study, it was assumed that there is only one strain of BFDV.

Failed attempts have been made to cultivate BFDV *in vitro* using tissue/cell culture and embryonated eggs (Johne *et al.*, 2004) thereby hindering the development of conventional vaccines. The availability of new vaccine technologies such as sub-unit

and DNA vaccines is therefore an attractive option for preventing PBFD in psittacines. DNA vaccination involves inoculation with an expression vector that encodes an antigenic protein; the encoded antigen is then produced *in situ* and elicits an immune response (Smith and Klinman, 2001). The basic requirements for the backbone of a plasmid DNA vector are:

- A strong eukaryotic promoter such as the cytomegalovirus (CMV) or simian virus 40 (SV40) promoters for high expression levels
- A cloning site for the insertion of the gene of interest
- A polyadenylation (polyA) sequence such as the bovine growth hormone (BGH) or SV40 polyA sequence to provide stabilisation of messenger RNA (mRNA)
- A selectable marker such as the ampicillin or kanamycin resistance genes for selection of positive transformants and
- A bacterial origin of replication such as *Escherichia coli* ColE1 for high copy numbers (Garmory *et al.*, 2003).

The vector pcDNA™3.1D/V5-His-TOPO® from Invitrogen was chosen for the purpose of this study as it possesses all the requirements of a DNA vaccine plasmid backbone while increasing the efficiency with which the antigenic gene is directionally inserted into the cloning site. As indicated in Figure 3.1.1, the vector pcDNA™3.1D/V5-His-TOPO® carries a CMV promoter to allow efficient, high-level expression of the recombinant antigenic protein, a directional TOPO® Cloning site that allows directional cloning of the PCR products in frame with a V5 epitope and polyhistidine C-terminal tag and a BGH polyA site that allows efficient transcription termination and stabilisation of the mRNA. The vector also has a V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) that allows detection of the recombinant protein with Anti-V5 antibodies while the C-terminal polyhistidine tag allows purification of the recombinant protein on metal-chelating resin as well as allowing detection of the protein with Anti-His antibodies. High copy number replication in *E. coli* is driven by the pUC origin and positive transformants are selected using ampicillin resistance.
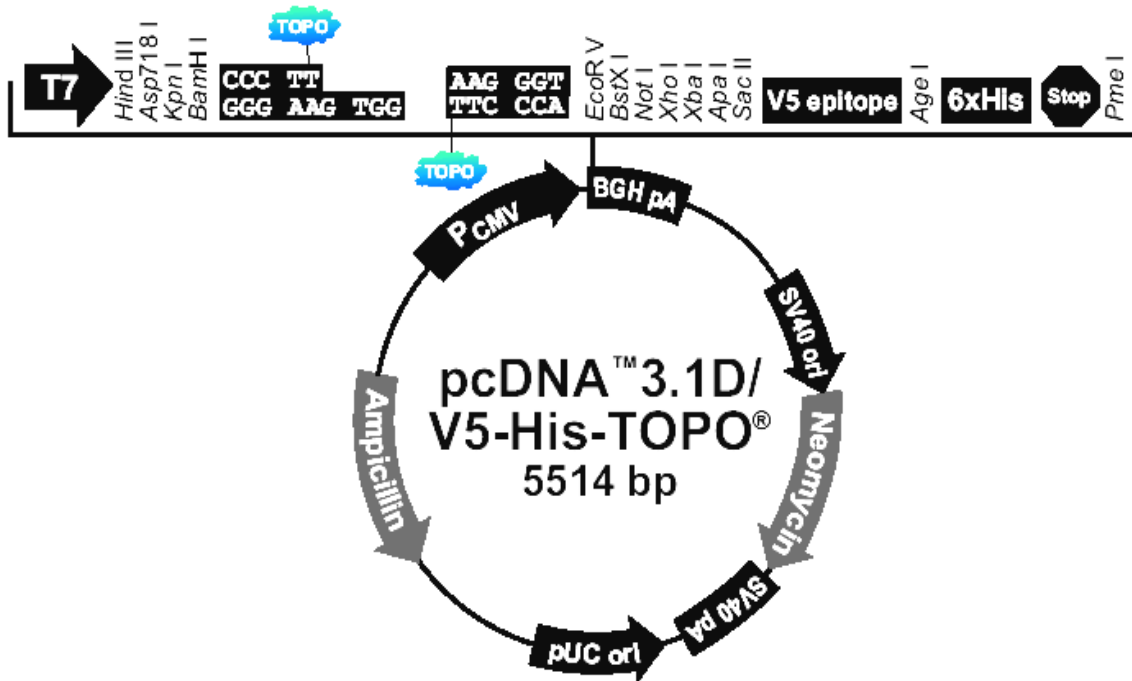
Figure 3.1.1: Schematic map showing the elements of the pcDNA™3.1D/V5-His-TOPO® vector. www.invitrogen.com

Topoisomerase I from *Vaccinia* virus binds to double stranded DNA (ds-DNA) at specific sites. It cleaves the phosphodiester bond after 5'-CCCTT in one strand, conserving the energy from the broken bond by forming a covalent bond between the 3' of the phosphate of the cleaved strand with its own tyrosyl residue [Tyr-274] (Shuman, 1994). The phospho-tyrosyl bond between the DNA and topoisomerase I can be attacked by the 5' hydroxyl of the original cleaved strand thereby reversing the reaction and releasing the enzyme. Invitrogen's TOPO® Cloning exploits this reaction allowing directional cloning to occur and thereby increasing the efficiency with which the gene of interest is inserted directionally into the cloning site.

Directional joining of ds-DNA using TOPO®-charged oligonucleotides occurs by adding four additional bases (CACC) to the incoming DNA which is then invaded by the 3' single-stranded end (overhang) GTGG in the vector [Figure 3.1.2]. The overhang anneals to the added bases i.e. CACC and stabilises the PCR product in the correct orientation. The inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.
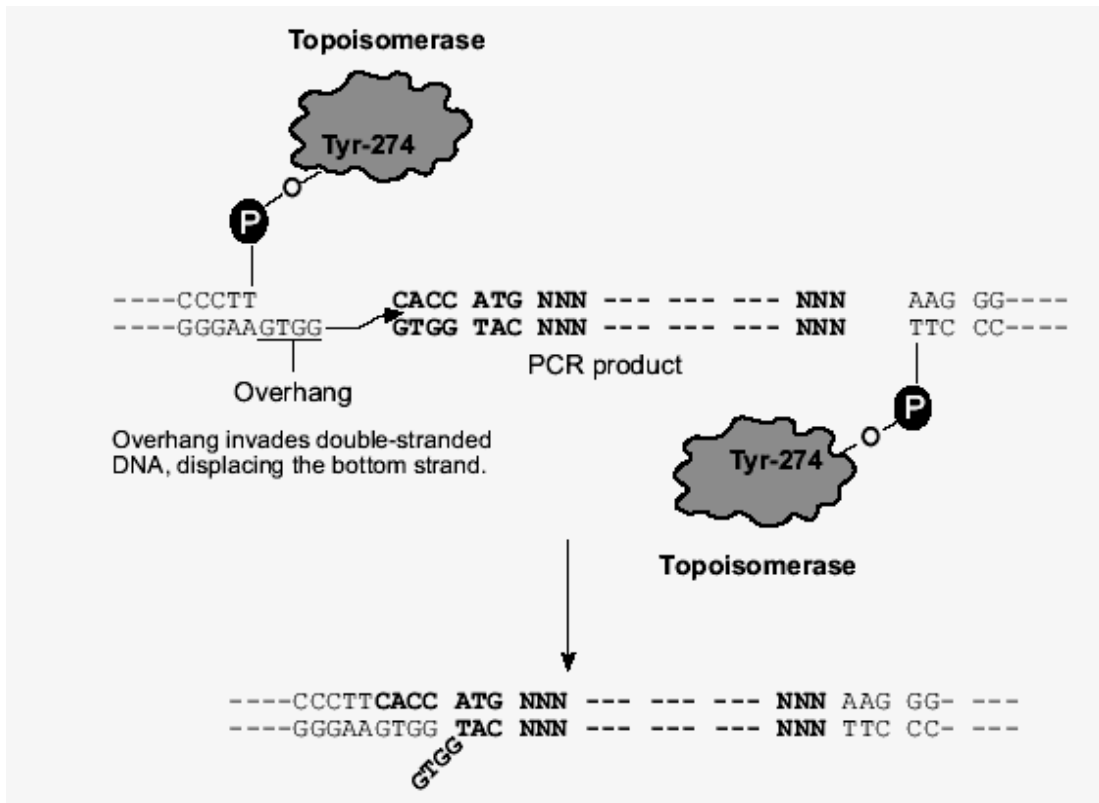
Figure 3.1.2: Directional TOPO® Cloning. www.invitrogen.com

It thus became the aim of this part of the research to develop a DNA vaccine candidate using the vector pcDNA™3.1D/V5-His-TOPO® that would have the potential to stimulate an immune response when administered to psittacines for the prevention of PBFD and to test if such an immune response occurs using a competitive ELISA.

3.2. MATERIALS AND METHODS.

3.2.1. <u>AMPLIFICATION OF COAT PROTEIN GENE BY POLYMERASE CHAIN REACTION.</u>

The entire gene encoding the CP of a BFDV isolate which was identified from a ring-necked parakeet in Bloemfontein using PCR amplification of the Rep gene was amplified to produce blunt-end PCR products in a total reaction volume of 50 µl. Reactions consisted of 5 µl of DNA template, 0.5 µl each of 100 mM forward and reverse primers (designed in the current study and shown in Table 3.1), 1 µl of 10 mM dNTP mix, 5 µl of 10X *Pfu* PCR buffer and 0.5 µl of *Pfu* polymerase made up to 50 µl with sterile Milli-Q water. DNA template was omitted in the negative control reaction.

Table 3.1: Primers used to amplify the CP gene of BFDV.

Primer	Size (bp)	Sequence
VACC F1	26	5'- CAC CCT GTG GGG CAC CTC TAA CTG CG -3'
VACC F2	27	5'- CAC CAT GCT GTG GGG CAC CTC TAA CTG -3'
VACC R1	26	5'- CTT AAG TGC TGG GAT TGT TAG GGG CA -3'
VACC R2	26	5'- AGT GCT GGG ATT GTT AGG GGC AAA CT -3'

The reverse primer VACC R1 was designed to include a stop codon while the reverse primer VACC R2 omits the stop codon to allow the expressed protein to carry a 6X C-terminal histidine tag encoded by the TOPO® vector. VACC F2 uses CTG as the start codon as present in the CP gene of BFDV while VACC F2 uses ATG as the start codon before the CTG of the CP gene. The reactions were performed in a Mastercycler Personal (Eppendorf®) and the conditions for the respective PCRs are indicated in Table 3.2. Initial denaturation was carried out at 95°C for 2 minutes (mins) and the cycle was repeated 30 times for each PCR. A final elongation step was carried out at 72°C for 5 mins.

A PCR was performed to amplify the control PCR product (750 bp) provided with the TOPO® vector in a reaction containing 1 µl of control DNA template, 5 µl of 10X *Pfu* PCR buffer, 0.5 µl of dNTP mix, 1 µl each of 0.1 µg.ml⁻¹ control PCR primers, 1 µl of *Pfu* polymerase made up to a total volume of 50 µl. The amplification parameters included an initial denaturation step at 94°C for 2 mins, denaturation at 94°C for 1

min, annealing at 55°C for 1 min, elongation at 72°C for 1 min repeated 25 times and a final elongation step at 72°C for 7 mins.

Table 3.2: PCR conditions used to amplify the CP gene of BFDV.

PCR	Primer Set	Conditions	Expected fragment (bp)
PCR 1	VACC F1/VACC R1	Denaturation = 94°C, 30 s	749
	VACC F1/VACC R2	Annealing = 58°C, 30s	745
		Elongation = 72°C, 2 mins	
PCR 2	VACC F2/VACC R1	Denaturation = 95°C, 30 s	752
	VACC F2/VACC R2	Annealing = 60°C, 30s	748
		Elongation = 72°C, 2 mins	

Amplified fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide. The PCR products were cleaned from solution using the Illustria™ DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions.

3.2.2. THE TOPO® CLONING REACTION.

To obtain optimal results a 2:1 molar ratio of PCR product:TOPO® vector was used in the TOPO® Cloning reaction. Table 3.3 shows the volumes added for each reaction carried out for transformation of chemically competent *E. coli* (supplied with the pcDNA™ 3.1 Directional TOPO® Expression Kit from Invitrogen).

Table 3.3: Reaction volumes for the TOPO® Cloning reactions to transform chemically competent *E. coli*.

Reagents	PCR Product VACC 1-1/VACC 1-2	PCR Product VACC 2-1/VACC 2-2	Vector only	Vector + control PCR Insert
PCR product	2 µl	1 µl	-	1 µl
Salt solution	1 µl	1 µl	1 µl	1 µl
Sterile water	2 µl	3 µl	4 µl	3 µl
TOPO® vector	1 µl	1 µl	1 µl	1 µl
Final volume	6 µl	6 µl	6 µl	6 µl

The reaction was mixed gently, incubated at rt for 5 mins and then placed on ice before proceeding to transformation.

3.2.3. TRANSFORMATION OF ONE SHOT® TOP10 COMPETENT CELLS.

One vial of One Shot® TOP10 chemically competent *E. coli* cells was thawed on ice per reaction. A volume of 2 µl from each TOPO® Cloning reaction was added to a vial of thawed cells and mixed gently by tapping briefly. The tube was incubated on ice for 30 mins, heat-shocked at $42^{o}C$ for 30 s and immediately transferred to ice. Room temperature S.O.C. medium (supplied with the pcDNA™ 3.1 Directional TOPO® Expression Kit) was added (250 µl) to the tube, the tube was capped and shaken horizontally (200 revolutions per minute [rpm]) at $37^{o}C$ for 1 hour (hr). After incubation, 50-200 µl from each transformation was spread-plated on a pre-warmed LB plate supplemented with ampicillin, X-gal (5-Bromo-4-chloro-3-indolyl-A-D-galactopyranoside) and IPTG (Isopropyl-2-D-thiogalactopyranoside) and incubated overnight at $37^{o}C$.

3.2.4. ANALYSIS OF TRANSFORMANTS.

White colonies (as a result of possible gene insertion into the cloning site thereby disrupting the *lac*Z ORF) were picked and cultured overnight on a shaker in 5 ml LB broth supplemented with 50 µl of ampicillin (10 mg.ml^{-1}) at $37^{o}C$. Plasmid DNA was isolated using the lysis by boiling method, microcentrifuge tube was filled with overnight culture and the cells pelleted by centrifugation at full speed for 30 s. The supernatant was aspirated and 350 µl of STET buffer (10 mM Tris, pH 8.0, I mM EDTA, pH 8.0, 0.1 M NaCl, 5% Triton X-100) added. The pellet was resuspended by vortexing, 25 µl of lysozyme (25 mg.ml^{-1}) was added and the tube vortexed for 3 s. The tube was transferred immediately to a boiling water bath for exactly 40 s and centrifuged at full speed for 10 mins. Cellular debris was removed using a sterile tooth pick then 40 µl of sodium acetate (2.5 M $CH_3COONa_3.H_2O$, pH 5.2) and 420 µl of isopropanol was added and the tube incubated at rt for at least 5 mins after vortexing to precipitate DNA. The tube was centrifuged in an Eppendorf 5417R at full speed for 5 mins at $4^{o}C$, the supernatant was aspirated and 1 ml of ice-cold 70% ethanol was added. The tube was centrifuged at full speed for 2 mins at $4^{o}C$, the supernatant aspirated and the pellet dried in a Speedvac Concentrator (SAVANT) for ~10 mins. The dried pellet was resuspended in 50 µl of TE-RNase buffer and incubated in a $37^{o}C$ water bath for 30 mins to digest contaminating RNA. The tube

was vortexed and DNA analysed or stored at -20°C until required for downstream applications.

The plasmid DNA was purified from solution as described previously (section 2.2.2, chapter 2) and analysed by restriction analysis using the restriction endonuclease *Bam*HI to confirm the presence and the correct orientation of the inserts. Cleaved products were visualised under UV illumination on a 1% agarose gel that was stained with ethidium bromide. Glycerol stocks of positive plasmids were prepared by resuspending cell pellets in 50% glycerol and stored at -20°C.

3.2.5. <u>TRANSFORMATION OF XL-10 GOLD COMPETENT CELLS.</u>

XL-10 gold competent *E. coli* cells (Stratagene) were transformed with 2 µl of plasmid DNA (pcDNA™3.1D/V5-His containing PCR products from the different primer combinations referred to as VACC 1-1, VACC 1-2, VACC 2-1, VACC 2-2 or pcDNA™3.1D/V5-His/*lac*Z) per transformation. The tube was incubated on ice for 30 mins, heat-shocked at 42°C for 30 s and transferred immediately to ice for 2 mins. Pre-warmed LB media (800 µl), 100 µl of 1 M glucose and 50 µl of 2 M Mg^{2+} was added and the tube incubated on a shaker at 37°C for 1 hr. The cells were pelleted in an Eppendorf 5415D at full speed for 30s, 900 µl of supernatant was aspirated and the pellet resuspended in the remaining 100 µl of supernatant. The cells were spread-plated on pre-warmed LB plates supplemented with ampicillin, X-gal and IPTG and the plates incubated overnight at 37°C.

Plasmid DNA was isolated using the lysis by boiling method (section 3.2.4) or the Eppendorf FastPlasmid™ Mini Kit or the EndoFree$^®$ Maxi/Mega Plasmid Kit (QIAGEN). Plasmid DNA was used in downstream applications and was sent to Inqaba Biotechnologies Ltd for sequencing. Sequences were analysed using Vector NTI Suite9 and DNAssist V.2.2 software.

3.2.6. <u>ISOLATION OF PLASMID DNA USING EPPENDORF FASTPLASMID™ MINI.</u>

White colonies were inoculated into 5 ml LB broth supplemented with 50 µl of 10 mg.ml^{-1} ampicillin and incubated overnight on a shaker at 37°C. A 2 ml microcentrifuge tube was filled with 1.5 ml overnight culture and the cells pelleted in

an Eppendorf 5415D at full speed for 1 min. The supernatant was aspirated and 400 µl of ice-cold Complete Lysis Solution was added. The sample was mixed thoroughly by constant vortexing at the highest speed for 30 s then incubated at rt for 3 mins. The sample was pipetted into a Spin Column Assembly, centrifuged at full speed for 1 min and 400 µl diluted Wash Buffer added. The column was centrifuged at full speed for 1 min, the filtrate decanted and the column placed back in the waste tube. The tube was centrifuged at full speed to dry the Spin Column, the column transferred to a Collection Tube and 50 µl of Elution Buffer was applied directly to the centre of the column membrane. The Collection Tube was capped over the Spin Column and centrifuged at full speed for 1 min, the column was discarded and the eluted DNA stored at – 20°C until required.

The presence and correct orientation of inserts was confirmed by restriction analysis using *Bam*HI and restricted fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide.

3.2.7. <u>ISOLATION OF ENDOTOXIN FREE PLASMID DNA.</u>

Endotoxin free DNA was prepared following the manufacturer's instructions for the EndoFree$^{®}$ Plasmid Maxi/Mega Kit (QIAGEN).

- Volumes, cartridges and tips for the Maxi Kit are indicated first followed by those used for the Mega Kit.

A volume of 5 µl of glycerol stock (VACC 1-1, VACC 1-2, VACC 2-1, VACC 2-2 or pcDNA™3.1D/V5-His/*lac*Z) was used to inoculate 5 ml LB broth supplemented with 50 µl of ampicillin (10 mg.ml^{-1}) as a starter culture. The starter culture was incubated with vigorous shaking (~ 300 rpm) at 37°C for 8 hrs. The starter culture was diluted 1/500 into LB broth supplemented with ampicillin (10mg.ml^{-1}). A volume of 200 or 1000 µl was inoculated into 100 or 500 ml of medium and grown overnight at 37°C for 16 hrs with vigorous shaking (~300 rpm). Bacterial cells were harvested by centrifugation in a Beckman Model J2-21 centrifuge (JA-20 rotor) at 6000 X *g* for 15 mins at 4°C. The bacterial pellet was resuspended in 10 or 50 ml of Buffer P1 containing RNase and LyseBlue, 10 or 50 ml of Buffer P2 was added and the sample mixed thoroughly by inverting 6 times until a homogenously blue suspension was attained. The sample was incubated at rt for 5 mins during which time a QIAfilter Maxi

Cartridge or QIAfilter Mega-Giga cartridge was prepared. A volume of 10 or 50 ml of chilled Buffer P3 was added to the lysate, mixed immediately and thoroughly by vigorously inverting 6 times until all trace of blue had disappeared and the suspension was colourless. The lysate was poured into the barrel of the QIAfilter Maxi cartridge or QIAfilter Mega-Giga cartridge and incubated at rt for 10 mins. For the QIAfilter Maxi cartridge, the cap was removed from the outlet nozzle, the plunger gently inserted into the cartridge and the lysate filtered into a 50 ml tube. For the QIAfilter Mega-Giga cartridge, the vacuum pump was switched on, the lysate filtered through, 50 ml of Buffer FWB2 was added to the cartridge and stirred with a sterile spatula then filtered through using the vacuum pump. Buffer ER (2.5 or 12.5 ml) was added to the filtered lysate, the lysate mixed thoroughly by inverting 10 times and incubated on ice for 30 mins. A QIAGEN-tip 500 or QIAGEN-tip 2500 was equilibrated by applying 10 or 35 ml of Buffer QBT to the column and allowing it to empty by gravity flow. The filtered lysate was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 X 30 or 200 ml Buffer QC and the DNA eluted with 15 or 35 ml of Buffer QN. The DNA was precipitated by adding 10.5 or 24.5 ml (0.7 volumes) of rt isopropanol to the eluted DNA which was then mixed and centrifuged immediately at ≥15 000 X g for 30 mins at 4°C. The supernatant was carefully decanted, the DNA pellet washed with 5 or 7 ml of endotoxin-free rt 70% ethanol and centrifuged at ≥15 000 X g for 10 mins. The supernatant was carefully decanted and the pellet air-dried for 10-20 mins. The DNA was redissolved in 100 or 500 µl of endotoxin-free Buffer TE. The DNA concentration was measured in an Eppendorf Biophotometer and stored at -20°C until required for downstream applications.

The presence and correct orientation of inserts was confirmed by restriction analysis using *Bam*HI and restricted fragments were observed under UV illumination on a 1% agarose gel stained with ethidium bromide as well as by sequencing samples at Inqaba Biotechnologies Ltd. Sequences were analysed and assembled using DNAssist v2.2 and Vector NTI Suite9.

3.2.8. <u>CELL CULTURE OF CHINESE HAMSTER OVARY CELLS.</u>

Chinese hamster ovary (CHO) cells were used for transfection studies and were either supplied by Miss Corné Kleyn (National Control Laboratories [NCL]) or cultured in the laboratory. Two vials of CHO KI cells (1999 cultures) were received from Miss

Kleyn, thawed rapidly in a 37°C water bath and added to a flask containing 10 ml growth medium (RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum [FBS] and 1X antibiotic/antimycotic from GIBCO). The flasks were incubated at 37°C and examined daily until cells were 100% confluent. When 100% confluency was reached, the growth medium was decanted, the monolayer rinsed 3 times with 10 ml of pre-warmed phosphate buffered saline (PBS) without calcium or magnesium ions and 1 ml pre-warmed Trypsin-EDTA (0.25%) added to a flask. The flask was rocked to cover the monolayer and incubated at 37°C for 2 mins to allow the cells to detach. Nine millilitres of growth media was added to the flask and the cells pipetted up and down gently to break up cell clumps. A cell scraper was used to remove the remaining adherent cells, the cells were transferred to a centrifuge tube and pelleted at 1800 rpm for 5 mins. The supernatant was discarded and the pellet resuspended in 8 ml of growth medium (CHO subculture ratio is 1:8) by tapping the tube and pipetting up and down. A volume of 10 ml growth medium was added to each of 8 flasks, 1 ml of the resuspended cells was added to each flask , the flask tapped 3 times to break off any extra clumps and incubated at 37°C until 100% confluent.

Alternatively, the pelleted cells were used to perform a cell count in order to plate out the required number of cells for performing transfections in 6-well plates. The day before transfection, the cells were trypsinised as above but the pellet was resuspended in 10 ml of growth medium (with FBS, without antibiotics); 1 ml of this suspension was added to 0.5 ml of trypan blue in PBS and mixed. A counting chamber was filled and the cells counted. Once the appropriate cell count had been reached, the cells were diluted in the appropriate volume of growth medium (with FBS, without antibiotics) required to give a final cell concentration of 1 X 10^5 cells/ml and 2 X 10^5 cells/ml. Each well on a 6-well plate was plated out with 2 ml of the diluted cells so that on the day of transfection (the next day) a density of 50-80% confluency was achieved.

3.2.9. <u>TRANSFECTION USING PolyFect$^{\circledR}$ TRANSFECTION REAGENT.</u>

Six well plates that were ~80% confluent containing 4 X 10^5 cells in 3 ml of growth medium were received from Miss Corné Kleyn and transfected using PolyFect Transfection Reagent (QIAGEN) according to the manufacturer's instructions. DNA suspended in TE buffer (minimum concentration of 0.1 µg.µl^{-1}) was diluted in growth medium (without FBS, proteins or antibiotics) to give a concentration of 1.5 µg in a

total volume of 100 µl. The DNA was mixed and centrifuged for a few seconds to remove drops from the top of the tube. PolyFect Transfection Reagent (10 µl) was added to the DNA solution, mixed by pipetting up and down 5 times and incubated at rt for 10 mins to allow complex formation. The growth medium was gently aspirated from the 6-well plate, the cells were washed once with 4 ml of pre-warmed PBS and 1.5 ml of fresh growth medium (containing FBS and antibiotics) was added. A volume of 0.6 ml of growth medium was added to the tube containing the transfection complexes, mixed by pipetting up and down twice and immediately transferred entirely to a well. The plate was gently swirled to ensure uniform distribution of the complexes and the plate incubated in a modular incubator chamber (Lasec) containing approximately 5% CO_2 in a 37°C incubator for 24-96 hrs.

3.2.10. <u>TRANSFECTION USING FuGENE 6 TRANSFECTION REAGENT.</u>

Six-well plates that were ~80% confluent containing 1 X 10^5 cells/ml or 2 X 10^5 cells/ml were transfected with FuGENE 6 Reagent (ROCHE) according to the manufacturer's instructions. For initial optimization, three ratios of FuGENE 6 Reagent (µl):DNA (µg) [3:1, 3:2, and 6:1] were used for transfections using the positive control plasmid DNA, pcDNA™3.1D/V5-His/*lac*Z supplied with the TOPO$^®$ vector.

Table 3.4 indicates the different volumes used for optimization of transfections using the positive control plasmid. One well was used as a cell control and did not contain any FuGENE 6 Reagent or DNA. FuGENE 6 Reagent was diluted in serum-free medium (without antibiotics) by pipetting directly into the medium. The tube was flicked to mix and incubated at rt for 5 mins. DNA was added to the diluted FuGENE 6 Reagent, the tube was tapped to mix the contents and incubated at rt for at least 15 mins. The entire volume of complex was added to a well and the plate incubated in a modular incubator chamber (Lasec) containing approximately 5% CO_2 in a 37°C incubator for 72 hrs.

Table 3.4: Compositions of reactions for optimization studies for transfections of CHO cells.

FuGENE 6:DNA	Serum-free media (µl)	FuGENE 6 Reagent (µl)	DNA (µg)
3:1	97	3	1
3:2	97	3	2
6:1	94	6	1

A 6:1 ratio of FuGENE 6 Reagent:DNA was found to be optimal and used for all subsequent transfections using VACC 1-1, VACC 1-2, VACC 2-1, and VACC 2-2.

3.2.11. <u>HARVEST OF CHINESE HAMSTER OVARY CELL EXPRESSED PROTEIN.</u>

CHO cells were harvested post-transfection using the CelLytic™-M mammalian cell lysis/extraction reagent (SIGMA). The growth medium was aspirated and the cells washed once with 4 ml of PBS. The PBS was discarded and 400 µl of CelLytic™-M (with protease inhibitor cocktail) was added. The cells were incubated on a shaker at rt for 15 mins then scraped off using a cell scraper. Cellular debris was pelleted by centrifugation of lysed cells in an Eppendorf 5415D at 12 000-20 000 X g for 15 mins. The protein containing supernatant was transferred to a sterile microcentrifuge tube and used in a ß-galactosidase assay or stored at -20°C for downstream applications. Beta-galactosidase containing samples were assayed for activity by incubating samples made up to 900 µl with water and 4 mg.ml^{-1} ONPG (o-nitrophenyl-ß-D-galactopyranoside) at 28°C for 5 mins. A volume of 200 µl of pre-warmed ONPG was added to the sample and the yellow colour reaction allowed to develop. The reaction was stopped when desired using 0.5 ml of 1M Na_2CO_3 and absorbance readings taken at a wavelength of 420 nm.

3.2.12. <u>POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTS.</u>

Protein samples were loaded on 10% SDS-PAGE gels after boiling in Laemmli sample buffer (with ß-mercaptoethanol) for 10 mins as described in section 2.2.9 (chapter 2). A maximum volume of 25 µl per sample and 5 µl of PageRuler™

Prestained Protein Ladder (Fermentas) was loaded. Gels were either stained overnight with PageBlue™ Protein Staining Solution (Fermentas) or blotted overnight. Proteins were detected on the membrane using chemiluminescence detection of the 6XHis tag (SuperSignal$^®$ West HisProbe™ kit described in section 2.2.10 of chapter 2) or the V5 epitope.

3.2.13. CHEMILUMINESENT DETECTION USING Anti-V5-HRP ANTIBODY.

Proteins expressed in frame with the V5 epitope of the TOPO$^®$ vector were detected using the Anti-V5-HRP antibody (Invitrogen). The membrane was blocked in 10 ml of PBST-5% non-fat, dry milk for 1 hr with shaking at rt then washed twice in 20 ml of PBST (PBS with 0.05% Tween-20) for 5 mins each. Anti-V5-HRP antibody was diluted 1:5000 in a tray with 10 ml of blocking buffer and the membrane placed in it and incubated for 1 hr at rt with shaking. The membrane was transferred to a clean tray and washed twice with 20 ml of PBST for 5 mins each. The blot was incubated with 10 ml of SuperSignal$^®$ West Pico Substrate Working Solution (PIERCE) for 5 mins then placed in a membrane sheet protector. It was placed in a film cassette and exposed to film for 60 s. The film was developed and the blot re-exposed for various time lengths.

3.2.14. PRELIMINARY VACCINE TRIAL USING VACC 2-2.

A vaccine trial (Animal Experiment number 13/07) using *Melopsittacus undulatus* (budgerigars) was carried out to test the effectiveness of the DNA vaccine, VACC 2-2, at producing any immune response. The budgerigars were inoculated with a total of 50 µg of VACC 2-2 or 10 µg of purified recombinant CP intramuscularly on each thigh as indicated in Table 3.5 and Figure 3.2.1 A. The recombinant CP was received from UCT and was expressed using the Bac-to-Bac baculovirus expression system (Invitrogen) and Sf-21 cells and purified using a HisTrap HP affinity chromatography column (Amersham Biosciences) according to Heath *et al*. (2006). Two budgerigars were used as a control group and received nothing while three birds were vaccinated with VACC 2-2 in endotoxin free saline. A second vaccinated group of 3 birds was administered VACC 2-2 in Freund's incomplete adjuvant while the last group of 2 birds was vaccinated with purified CP in Freund's incomplete adjuvant.

The vaccinated birds received a booster inoculation with the same formulation as the prime 4 weeks later.

[A]

[B]
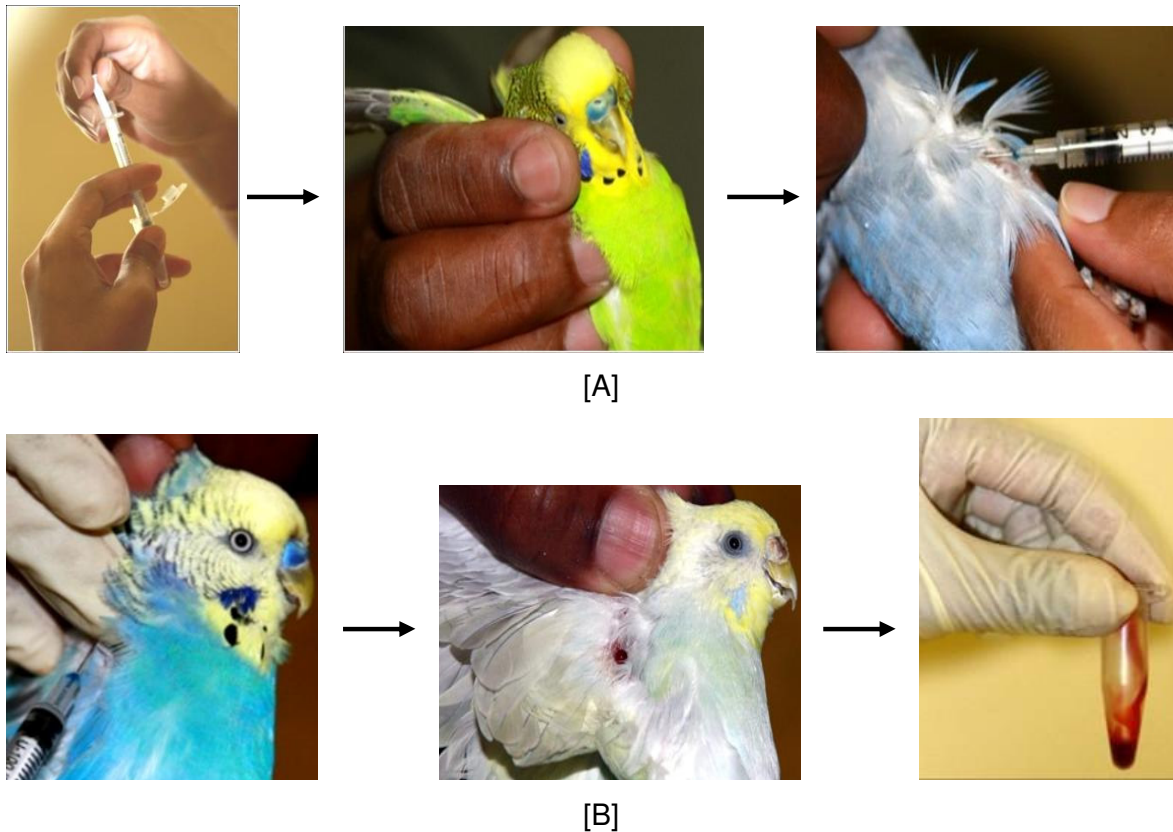
Figure 3.2.1: Vaccination [A] and bleeding [B] of *Melopsittacus undulatus* (budgerigars).

Prior to vaccination, blood samples were taken to determine if the parrots were negative for BFDV antibodies using the indirect competitive ELISA established in this study (section 2.2.14, chapter 2) [Figure 3.2.1 B]. Blood samples were collected 28 and 42 days post-vaccination. Sera was collected from the blood and stored at -20°C until tested for detectable antibodies to BFDV CP using the indirect competitive ELISA.

Table 3.5: Table indicating the different formulations of vaccine administered to *Melopsittacus undulatus* (budgerigars) in a preliminary trial study.

Bird	Sex	Vaccine formulation
1	Male	Nil
2	Female	Nil
3	Male	VACC 2-2 in saline
4	Female	VACC 2-2 in saline
5	Male	VACC 2-2 in saline
6	Male	VACC 2-2 in Freund's incomplete adjuvant
7	Male	VACC 2-2 in Freund's incomplete adjuvant
8	Male	VACC 2-2 in Freund's incomplete adjuvant
9	Male	Purified CP in Freund's incomplete adjuvant
10	Male	Purified CP in Freund's incomplete adjuvant

3.2.15. <u>SUMMARY OF EXPERIMENTAL DESIGN.</u>

A summary of the major steps carried out in this part of the research study is shown in Figure 3.2.2.
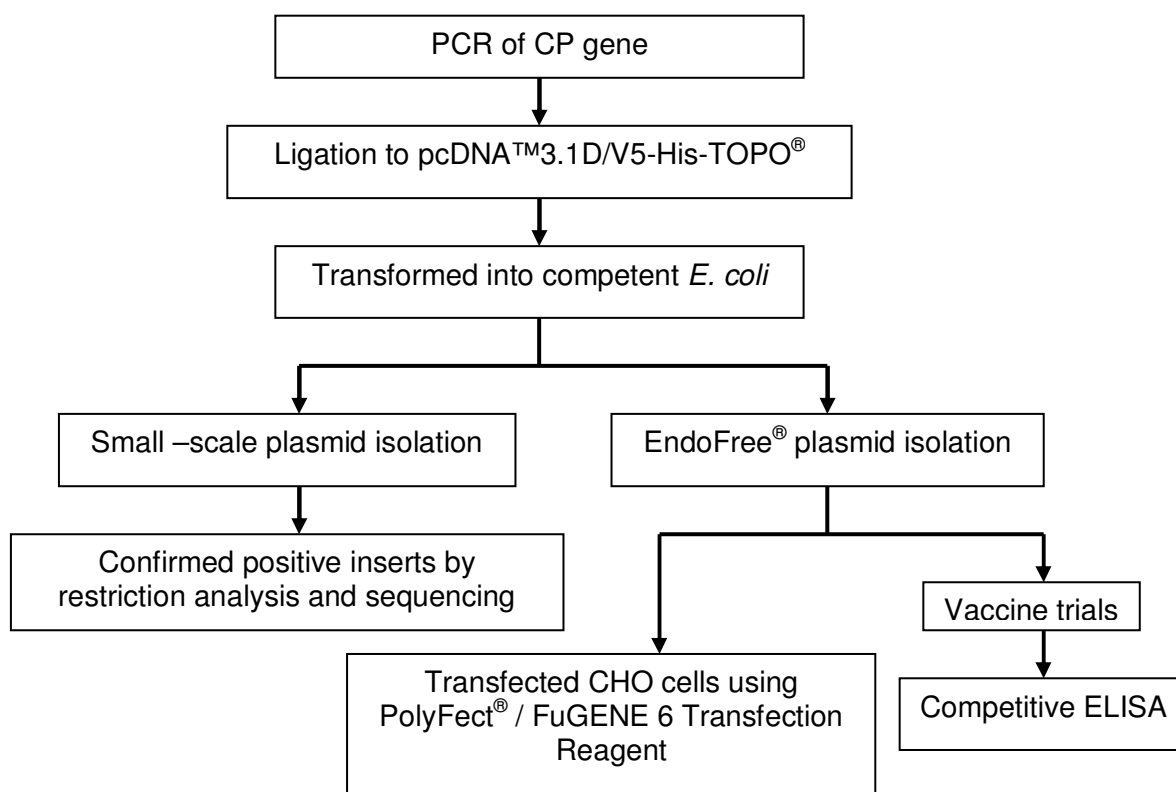
Figure 3.2.2: Flow diagram of the important steps carried out towards developing a DNA vaccine candidate.

3.3. RESULTS AND DISCUSSION.

3.3.1. <u>PCR AND RESTRICTION PROFILES FROM PRIMER SETS VACC F1/VACC R1 AND VACC F1/VACC R2.</u>

The first step when developing a DNA vaccine is to correctly identify the gene of interest and to amplify it. The sequence for the entire CP gene (GenBank accession number AF080560) was used to design the primers to amplify this gene. The CP gene consisted of 744 nucleotides so the expected size of the PCR product using primer set VACC F1/VACC R1 was 749 base pairs (bp) and the expected size of fragment from primer set VACC F1/ VACC R2 was 745 bp. Figure 3.3.1 indicates the sizes of the PCR products obtained from primer sets VACC F1/VACC R1 and VACC F1/VACC R2.
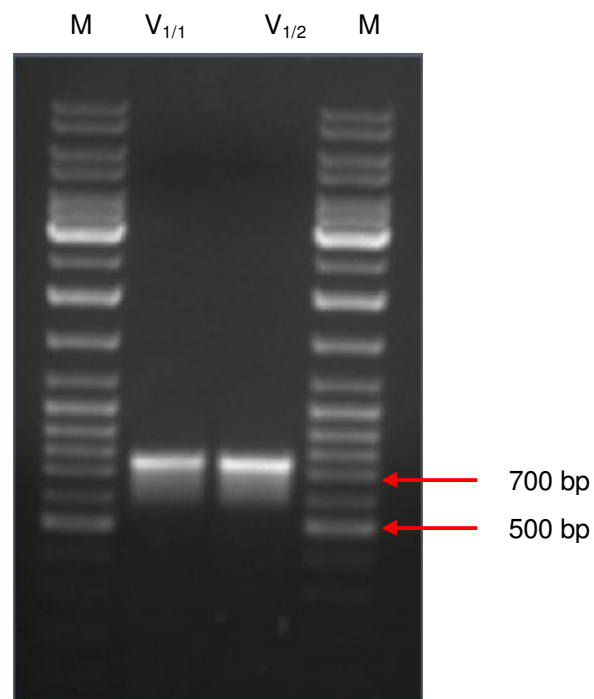
Figure 3.3.1: A 1% w/v agarose gel visualized under UV illumination separating the PCR products obtained from primer sets VACC F1/VACC R1 (Lane $V_{1/1}$) and VACC F1/VACC R2 (Lane $V_{1/2}$). Lane M represents the Fermentas O' GeneRuler™ DNA Ladder Mix molecular weight marker.

PCR products from reverse primer VACC R1 included a stop codon that prevented the addition of a 6X C-terminal histidine tag and the V5 epitope encoded by

pcDNA™3.1D/V5-His-TOPO® during translation while PCR products from reverse primer VACC R2 omitted the stop codon to allow the addition of the 6X C-terminal histidine tag and V5 epitope encoded by the vector during translation.

The PCR products were purified from solution and used in the TOPO® Cloning reaction where they were successfully ligated to the vector pcDNA™3.1D/V5-His-TOPO®. The ligated vector was used to transform competent TOP 10 *E. coli* cells and small scale plasmid isolations were performed. The plasmid DNA was cleaved using the restriction endonuclease *Bam*HI to confirm the presence of inserts in the correct orientation. Plasmid DNA in the correct orientation results in two fragments of 5661 bp and 602 bp when cleaved with *Bam*HI and is represented in Figure 3.3.2 A. Transformation of competent XL-10 Gold *E. coli* with the same vector also yielded the same restriction fragment length polymorphism (RFLP) pattern [Figure 3.3.2 B].
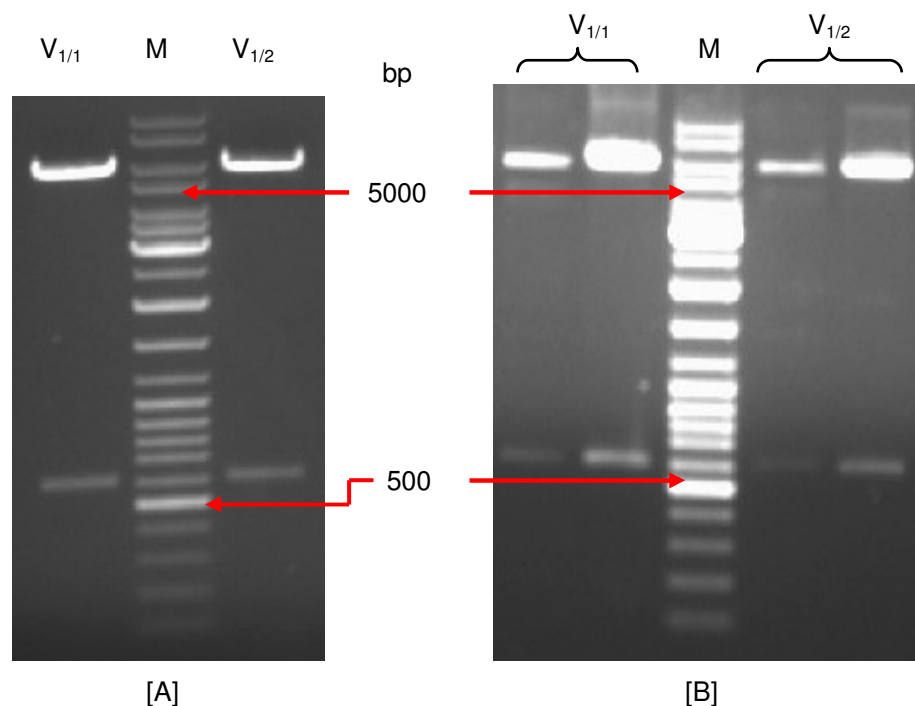
Figure 3.3.2: 1% w/v agarose gels visualised under UV illumination showing the expected RFLP profile of VACC 1-1 (Lanes $V_{1/1}$) and VACC 1-2 (Lanes $V_{1/2}$) isolated from competent TOP10 [A] and XL-10 Gold [B] *E. coli* when cleaved with *Bam*HI. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

Transformation of competent XL-10 Gold *E. coli* using the positive control vector pcDNA™3.1D/V5-His/*LacZ* was also performed and small scale plasmid isolations

carried out. Plasmid DNA was confirmed by double digest using *Bam*HI and *Eco*RI and the correct RFLP pattern (two fragments of 5553 and 3033 bp) was observed [Figure 3.3.3].
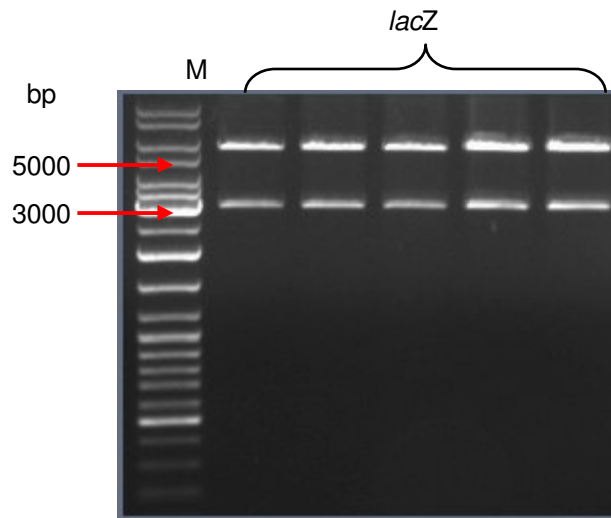
Figure 3.3.3: A 1% agarose gel visualized under UV illumination indicating the expected RFLP profile for pcDNA™3.1D/V5-His/*lac*Z (Lanes *lac*Z) when cleaved with *Bam*HI/*Eco*RI. Lane M represents the O'GeneRuler™ DNA Ladder Mix molecular weight marker.

pcDNA™3.1D/V5-His-TOPO® was selected for the highly efficient, 5 minute, one-step cloning strategy to directionally clone blunt-end PCR products into the vector. This directional cloning occurs at a greater than 90% efficiency which minimises the screening step and allows the gene of interest to be expressed directly in mammalian cell lines once it has been cloned, analysed and transfected. The forward primer in the study was designed to include a 5'-CACC to the PCR product to facilitate directional TOPO® cloning. The overhang (GTGG) on pcDNA™3.1D/V5-His-TOPO® invades the 5' end of the PCR product, anneals to the added bases and stabilises the PCR product in the correct orientation as was confirmed by RFLP using the restriction endonuclease *Bam*HI on the plasmids containing the CP gene inserts as well as the positive control plasmid: VACC 1-1, VACC 1-2 and pcDNA™3.1D/V5-His/*lac*Z .

3.3.2. <u>SDS-PAGE AND WESTERN BLOTS OF PolyFect® TRANSFECTED CHO CELLS WITH VACC 1-1 AND VACC 1-2.</u>

The next important step in DNA vaccine development after confirming the presence and correct sequence of inserts in the vector backbone was to transiently transfect

mammalian cells to verify the ability of the vector to direct protein expression after it enters the cell. Protein from the first transfection of the CHO cells using the plasmids pcDNA™3.1D/V5-His/*lacZ* (positive control) and VACC 1-1 and VACC 1-2 could not be successfully detected and therefore, optimization experiments had to be performed using the positive control plasmid. The transfection was repeated and ß-galactosidase assays and western blot analysis were performed for cell extracts from the CHO cells that had been transfected with the positive control that encodes a *lacZ* gene for expression of the enzyme. ß-galactosidase can be assayed colorimetrically using the substrate ONPG which is hydrolysed by the enzyme to yield o-nitrophenol, a yellow-coloured product that can be measured photometrically.

A protein band between 55 kDa and 70 kDa was detected in all the samples [Figure 3.3.4] that were collected at 48, 72 and 96 hrs post-transfection. Expressed ß-galactosidase from the pcDNA™3.1D/V5-His/lacZ vector is 120 kDa while recombinant CP is ~30 kDa in size due to the presence of the 6X His tag indicating that the protein bands obtained were incorrect and were considered contaminating protein bands. Furthermore no ß-galactosidase activity was observed.
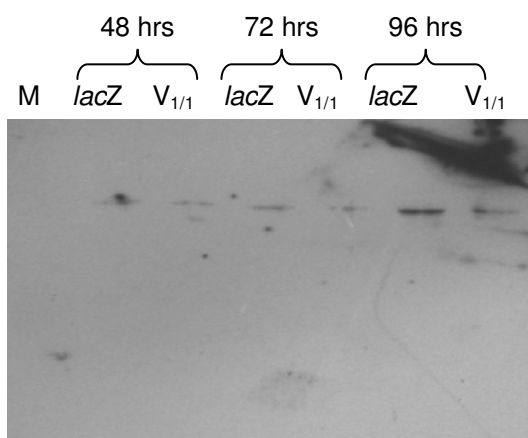
Figure 3.3.4: Protein bands detected on a western blot using chemiluminescence. Lanes *lacZ* and $V_{1/1}$ represent extracted protein from CHO cell lysates that were transiently transfected with the vectors pcDNA™3.1D/V5-His/*lacZ* and VACC 1-1, respectively in PolyFect® Transfection Reagent (QIAGEN). A protein band between 55 kDa and 70 kDa was observed in all the lysates that were collected 48, 72 and 96 hrs post-transfection. Lane M represents the PageRuler™ Prestained Protein Ladder (Fermentas).

Troubleshooting into the possible reasons for the lack of expression led to the probable identification of the problem which was the lack of use of endotoxin free

plasmid DNA. Until this point, the plasmid DNA was prepared using the FastPlasmid™ Mini kit from Eppendorf which does not isolate plasmid DNA as endotoxin free. Endotoxins or lipopolysaccharides are cell membrane components of Gram negative bacteria composed of a hydrophobic lipid A moiety, a complex array of sugar residues and negatively charged phosphate groups. These components give endotoxins hydrophobic, hydrophilic and charged regions which are capable of interacting with numerous other molecules. Endotoxins strongly influence the transfection of DNA into primary cell lines and sensitive cultured cells and additionally interfere with *in vitro* transfection into immune cells by causing non-specific activation of immune responses (Weber *et al.*, 1995).

It is therefore important for plasmid DNA as well as all plastic ware, media and sera to be free from endotoxin contamination in order to avoid misinterpretation of experimental results. The chemical structure and properties of endotoxin molecules and their tendency to form micellar structures lead to the copurification of endotoxins with plasmid DNA. Using the EndoFree® plasmid Kit from QIAGEN which integrates endotoxin removal into the standard QIAGEN Plasmid purification procedure, plasmid DNA was prepared as endotoxin free DNA and used to transfect CHO cells. Samples were collected after 48 and 72 hrs and a ß-galactosidase assay performed for the 72 hr samples as shown in Figure 3.3.5.

Time (min)	Absorbance (420 nm)		
	*lac*Z	VACC 1-1	Control
0	0.027	0.019	0.029
5	0.045	0.019	0.029
10	0.07	0.019	0.029
15	0.094	0.019	0.029
20	0.119	0.019	0.029
25	0.145	0.019	0.029
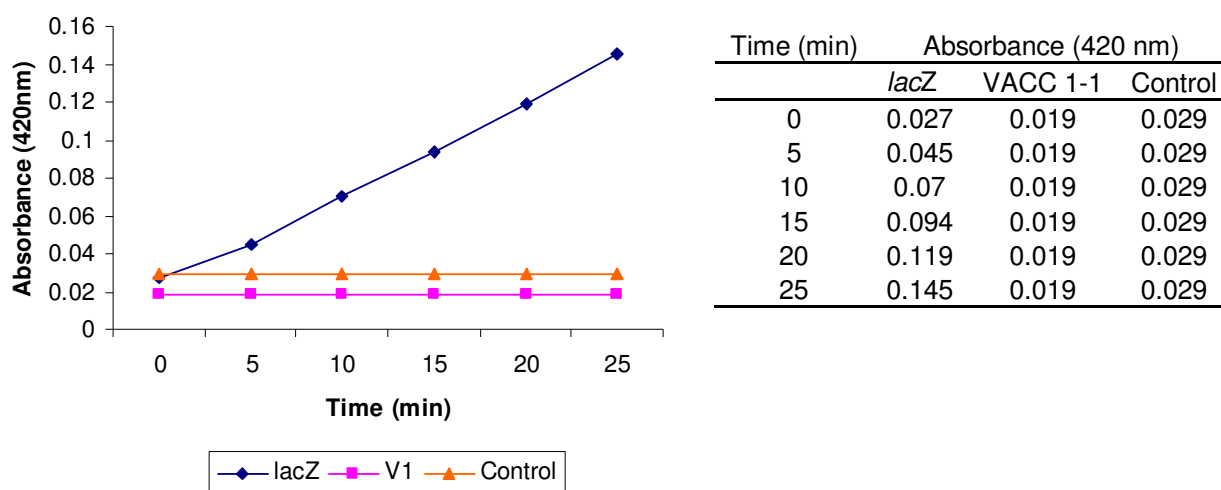
Figure 3.3.5: A graph showing the results of a ß-galactosidase assay performed on 72 hr protein samples collected from CHO cells transfected with endotoxin free pcDNA™3.1D/V5-His/*lac*Z and VACC 1-1 in PolyFect® Transfection Reagent and a negative control consisting of CHO cells only. The table indicates the absorbance readings obtained for the samples at a wavelength of 420 nm.

Western blot analyses were performed on the post-transfected 72 hr samples using chemiluminescent detection of the 6X His tag [Figure 3.3.6 A] or chemiluminescent detection of the V5 epitope [Figure 3.3.6 B]. A number of protein bands were detected in all the samples including the negative control using chemiluminescent detection of the 6XHis tag but these were bands of unexpected size. The 120 kDa ß-galactosidase protein band could not be detected using the His tag. However, as seen in Figure 3.3.6 B, a single protein band of the expected size, 120 kDa, was only observed in the lane representing the sample from the positive control i.e. ß-galactosidase while the other lanes did not exhibit any protein bands.
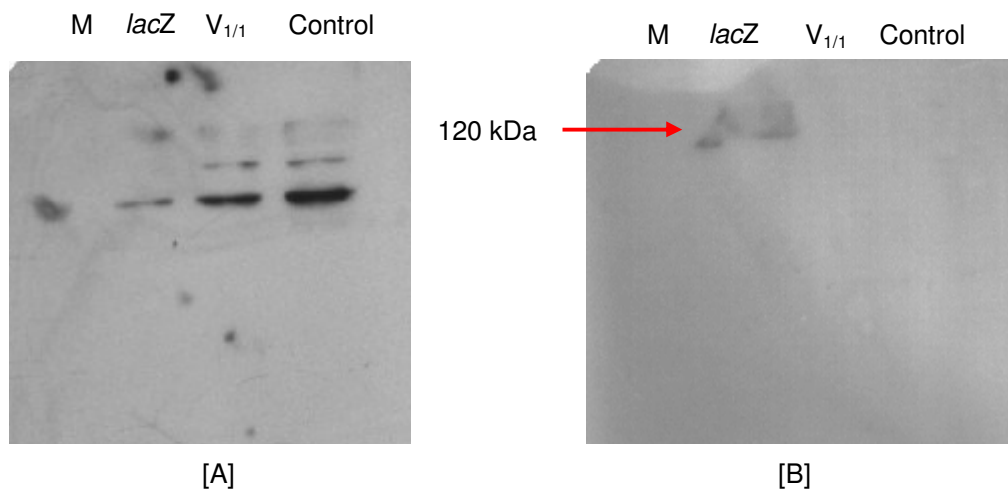
Figure 3.3.6: Protein bands detected on a western blot using chemiluminescent detection of the 6X His tag [A] and the V5 epitope [B]. Lanes *lac*Z and $V_{1/1}$ represent extracted protein from CHO cells transfected with pcDNA™3.1D/V5-His/*lac*Z and VACC 1-1, respectively in PolyFect® Transfection Reagent. Lane Control represents the negative control used during transfections and only consisted of CHO cells. Lane M represents the PageRuler™ Prestained Protein Ladder from Fermentas.

The detection of the additional non-specific protein bands using the SuperSignal® West HisProbe™ Kit (PIERCE) can be attributed to the use of HisProbe™-HRP which detects polyhistidine tags independent of location and adjacent sequences. Chemiluminescence using Anti-V5-HRP antibody (Invitrogen) on the other hand, is specific for the V5 epitope and therefore it can be concluded that in this instance chemiluminescent detection of the V5 epitope using Anti-V5-HRP antibody was more sensitive and specific than detection of the 6XHis tag.

Both the ß-galactosidase assay and chemiluminescent detection of the V5 epitope indicated that the concentration of recombinant protein being expressed was very low. The ß-galactosidase assay gave an A_{420} reading of only 0.07 after 10 mins and the Western blot showed a very faint band. Positive reports on the use of FuGENE 6 Transfection Reagent from Roche were received and it was decided to use this product as a transfection reagent instead of PolyFect® Transfection Reagent (QIAGEN) to determine if better expression levels could be achieved in the present study.

3.3.3. SDS-PAGE AND WESTERN BLOTS OF FuGENE 6 TRANSFECTED CHO CELLS WITH VACC 1-1 AND VACC 1-2.

Optimization reactions were carried out to determine a suitable ratio of FuGENE 6 Reagent:DNA using the positive control plasmid pcDNA™3.1D/V5-His/*lac*Z. Expression of recombinant enzyme was monitored using a ß-galactosidase assay and Western blot. The ß-galactosidase assay [Figure 3.3.7] yielded positive results for two ratios of FuGENE 6 Reagent:DNA, 3:1 and 6:1. Although some activity was observed for the 3:1 ratio it was significantly lower than that in the samples collected from the 6:1 ratio transfection as indicated by the A_{420} readings after 10 mins: cells transfected with a 3:1 ratio gave a reading of 0.039 while those transfected with a 6:1 ratio gave a reading of 1.158. The sample from the 3:2 ratio gave an A_{420} reading of 0.021 which was the same reading obtained for cells transfected with VACC 1-1 and the negative control consisting of untransfected cells, indicating that these samples did not have any measurable activity of expressed ß-galactosidase.

Time (min)	Absorbance (420 nm)				
	control	3:2 VACC 1-1	3:1 *lacZ*	3:2 *lacZ*	6:1 *lacZ*
0	0.024	0.021	0.025	0.021	0.25
1	0.024	0.021	0.027	0.021	0.402
2	0.024	0.021	0.029	0.021	0.5
3	0.024	0.021	0.03	0.021	0.596
4	0.024	0.021	0.031	0.021	0.68
5	0.024	0.021	0.033	0.021	0.772
6	0.024	0.021	0.034	0.021	0.851
7	0.024	0.021	0.035	0.021	0.936
8	0.024	0.021	0.037	0.021	1.012
9	0.024	0.021	0.038	0.021	1.091
10	0.024	0.021	0.039	0.021	1.158

Figure 3.3.7: A graph showing the results of a ß-galactosidase assay performed on 72 hr protein samples collected from CHO cells transfected with three ratios, 3:1, 3:2 and 6:1, of pcDNA™3.1D/V5-His/*lacZ* and 3:2 VACC 1-1 in FuGENE 6 Reagent and a negative control consisting of CHO cells only. The table indicates the absorbance readings obtained for the samples at a wavelength of 420 nm.

The results from the Western blot [Figure 3.3.8] correlate to the results from the ß-galactosidase assay as a clear 120 kDa protein band can be observed in the lanes with samples from the 3:1 and 6:1 ratios of *lacZ* with the band in the 6:1 lane being of the highest intensity. The ß-galactosidase band in the lane 3:1 is visibly intense but chemiluminescent detection on a Western blot is much more sensitive, detecting as little as 50 ng of protein depending on the probe used, than absorbance readings from activity assays.

Figure 3.3.8: ß-galactosidase bands detected on a Western blot using chemiluminescent detection of the V5 epitope from samples collected from CHO cells transfected with a 3:1, 3:2 and 6:1 ratio of FuGENE 6 Reagent:pcDNA™3.1D/V5-His/*lac*Z. Lanes 3:1 $V_{1/1}$ and Control represent samples collected from CHO cells transfected with a 3:1 ratio of FuGENE 6 Reagent:VACC 1-1 and untransfected cells, respectively. Lane M represents the PageRuler™ Prestained Protein Ladder from Fermentas.

The optimal ratio of FuGENE 6 Reagent:DNA was considered to be 6:1 as this ratio gave the highest concentration of recombinant ß-galactosidase and was used for all subsequent transfections. The CHO cells were then transfected with VACC 1-1, VACC 1-2 and pcDNA™3.1D/V5-His/*lac*Z using the 6:1 ratio of FuGENE 6 Reagent:DNA and lysed 72 hrs post-transfection to collect protein samples. A ß-galactosidase assay was once again performed to confirm that the *lac*Z gene was being expressed and Western blots performed to detect expressed CP. As expected, the samples from cells transfected with VACC 1-1 and VACC 1-2 as well as untransfected cells did not have any activity while positive activity was indicated in the sample from the cells transfected with the *lac*Z gene which had an A_{420} of 0.388 after 10 mins [Figure 3.3.9].

Time (min)	Absorbance (420 nm)			
	control	VACC 1-1	VACC 1-2	lacZ
0	0.337	0.316	0.314	0.307
1	0.337	0.316	0.314	0.313
2	0.337	0.316	0.314	0.32
3	0.337	0.316	0.314	0.332
4	0.337	0.316	0.314	0.344
5	0.337	0.316	0.314	0.354
6	0.337	0.316	0.314	0.359
7	0.337	0.316	0.314	0.368
8	0.337	0.316	0.314	0.376
9	0.337	0.316	0.314	0.384
10	0.337	0.316	0.314	0.388
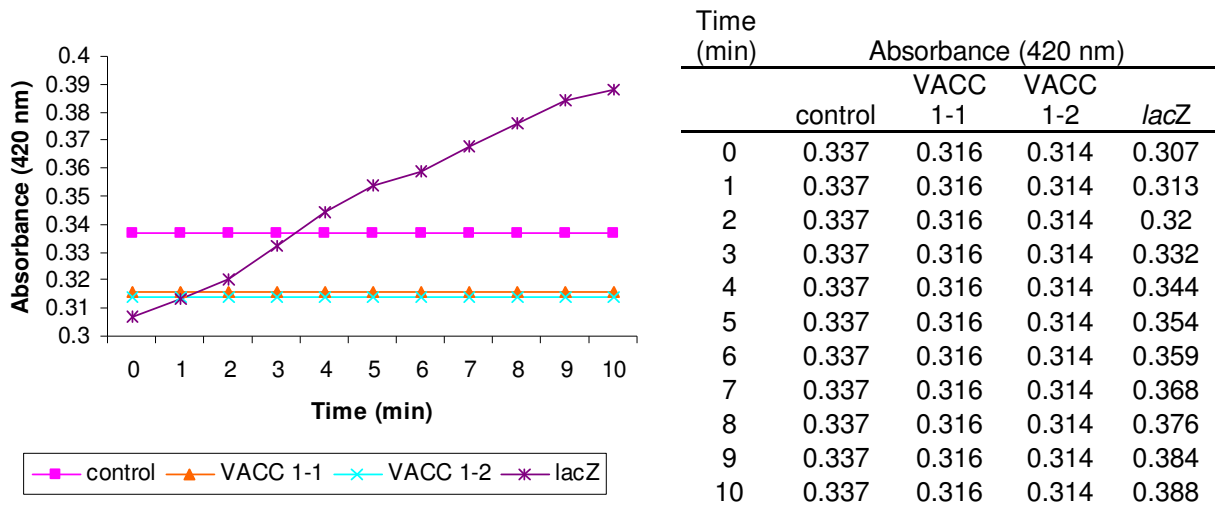
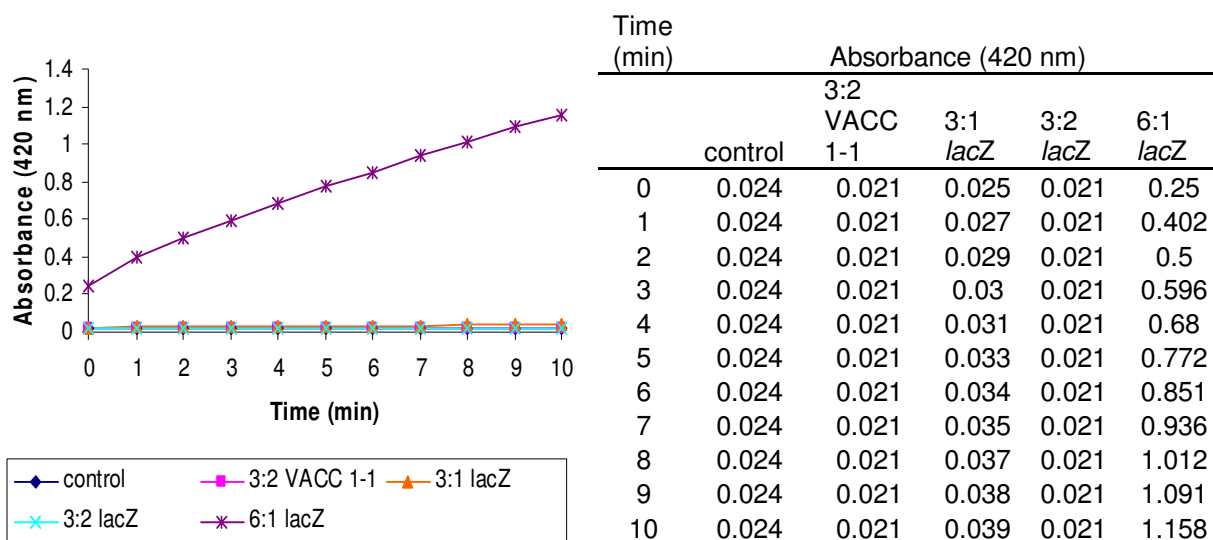**Figure 3.3.9:** A graph showing the results of a ß-galactosidase assay performed on 72 hr protein samples collected from CHO cells transfected with a 6:1 ratio of pcDNA™3.1D/V5-His/*lac*Z, VACC 1-1 and VACC 1-2 in FuGENE 6 Reagent and a negative control consisting of CHO cells only. The table indicates the absorbance readings obtained for the samples at a wavelength of 420 nm.

The ß-galactosidase protein band was detected on the Western blot; however no other protein bands of the expected size for recombinant CP (~30 kDa) were detected even when the Western blot was repeated [Figure 3.3.10 A and B]. Colorimetric detection on a Western blot using rabbit raised anti-BFDV and secondary conjugated anti-rabbit-alkaline phosphatase (anti-rabbit-AP) did not yield any specific protein bands at ~30 kDa either.
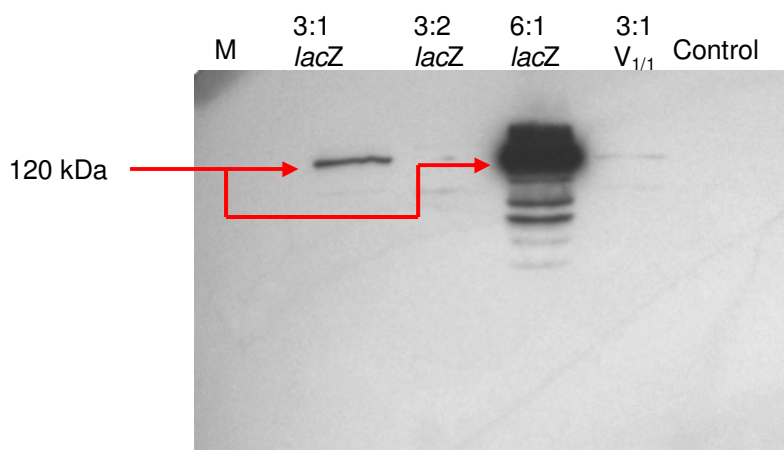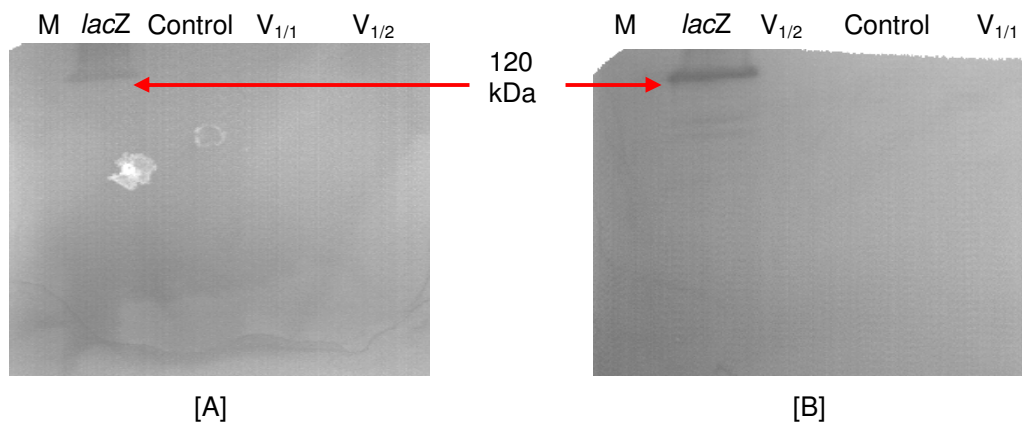
Figure 3.3.10: ß-galactosidase bands detected on a Western blot using chemiluminescent detection of the V5 epitope from samples collected from CHO cells transfected with a 6:1 ratio of FuGENE 6 Reagent:pcDNA™3.1D/V5-His/*lac*Z. Lanes $V_{1/1}$,$V_{1/2}$ and Control represent samples collected from CHO cells transfected with a 6:1 ratio of FuGENE 6 Reagent:VACC 1-1 and VACC 1-2 and untransfected cells, respectively. There were no visible bands of size ~30 kDa which is the expected size for recombinantly expressed CP. Lane M represents the PageRuler™ Prestained Protein Ladder from Fermentas.

The BFDV CP gene sequence that was used in the present study contains CTG as the start codon instead of the usual ATG and the forward primer VACC F1 was designed containing the former codon. No codon table is available for parrot species therefore the codon table for *Gallus gallus* (chicken) [http://www.kazusa.or.jp] was used indicating that the codon CTG codes for the amino acid lysine at the highest frequency and ATG for methionine. It is only ATG that codes for the amino acid methionine and because the CP gene was being expressed in eukaryotic cells it may be possible that expression was lacking due to the absence of the correct start codon. Differences between codon usage in a heterologous gene and the host organism may affect expression as observed in a DNA vaccine containing gp120 of human immunodeficiency virus type 1 (HIV-1) where a synthetic gp120 sequence in which most of the wild-type codons were replaced with codons from highly expressed human genes showed increased *in vitro* expression of gp120 compared to the wild-type sequence (Garmory *et al.*, 2003). In addition, significantly increased antibody titres and cytotoxic T cell (CTL) reactivity were observed following administration of the vector containing the synthetic sequence.

3.3.4. PCR AND RESTRICTION PROFILES FROM PRIMER SETS VACC F2/VACC R1 AND VACC F2/VACC R2.

A new forward primer was designed to include a start codon ATG before BFDV's start codon CTG (Table 3.1 indicates the complete sequence of the second forward primer VACC F2). PCR fragments of ~700 bp were observed on the gel and correlated to the expected sizes of 752 and 748 bp for PCR products obtained using the primer set VACC F2/VACC R1 and VACC F2/VACC R2, respectively [Figure 3.3.11 A]. A RFLP profile of cleaved PCR products with *Bam*HI yielded two fragments of the expected sizes of 590 bp and 184 bp [Figure 3.3.11 B].
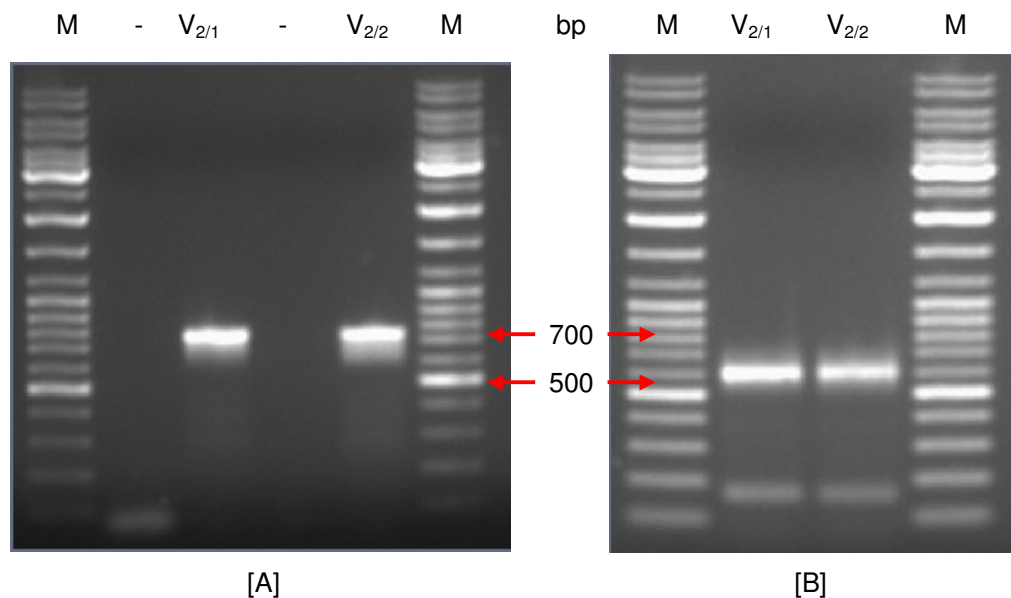
Figure 3.3.11: A 1% w/v agarose gel visualized under UV illumination separating the PCR products obtained from primer sets VACC F2/VACC R1 (Lane $V_{2/1}$) and VACC F2/VACC R2 (Lane $V_{2/2}$) [A]. A restriction profile of PCR products from the primer sets VACC F2/R1 and VACC F2/R2 using the restriction endonuclease *Bam*HI [B]. Lane M represents the Fermentas O'GeneRuler™ DNA Ladder Mix molecular weight marker.

The PCR products were then ligated to the pcDNA™3.1D/V5-His-TOPO® vector and transformed into competent *E. coli* cells. Inserts in the correct orientation were observed by cleaving isolated plasmid DNA with the restriction endonuclease *Bam*HI. Two fragments of 5645 bp and 621 bp were expected in the RFLP profile as indicated in Figure 3.3.12 A. The plasmid DNA was used to retransform competent *E. coli* cells and endotoxin free plasmid DNA prepared using the EndoFree® Plasmid Kit

(QIAGEN). Isolated endotoxin free plasmid DNA was once again cleaved using *Bam*HI to confirm the presence and orientation of inserts [Figure 3.3.12 B].
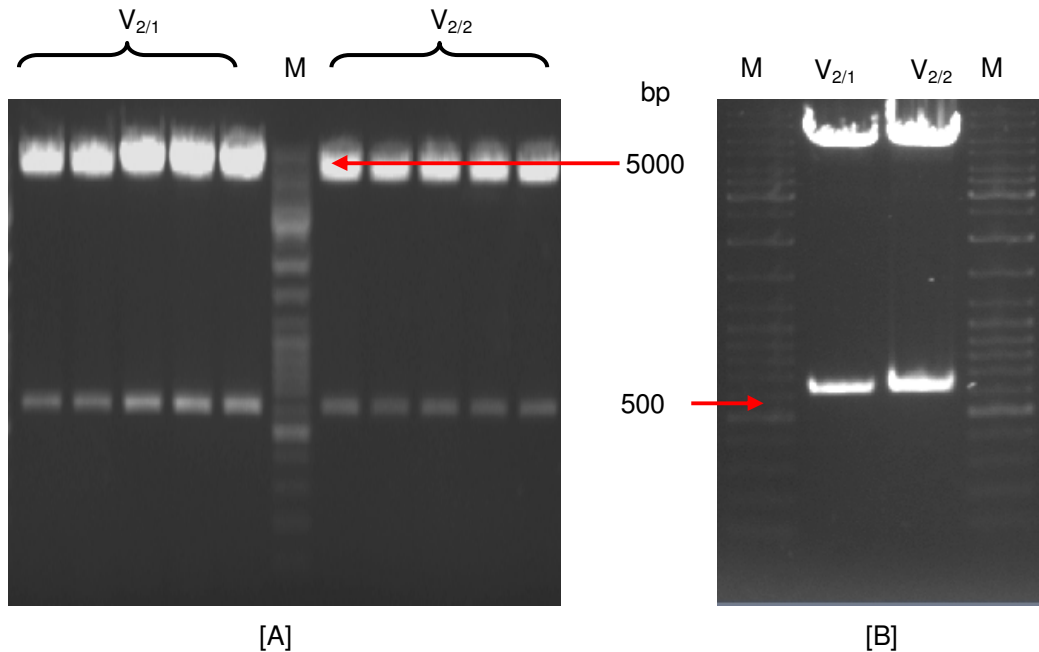
Figure 3.3.12: A restriction profile of plasmid DNA VACC 2-1 and VACC 2-2 cleaved with *Bam*HI [A and B]. Two fragments of ~5000 bp and ~600 bp are observed. Lane M represents the Fermentas O' GeneRuler™ DNA Ladder Mix molecular weight marker.

3.3.5. SDS-PAGE AND WESTERN BLOTS OF FuGENE 6 TRANSFECTED CHO CELLS WITH VACC 2-1 AND VACC 2-2.

A 6:1 ratio of FuGENE 6 Reagent:DNA was used to transfect CHO cells with pcDNA™3.1D/V5-His/*lac*Z, VACC 2-1 and VACC 2-2. Western blots were performed on cell lysates collected 72 hrs post transfection but the only protein bands observed was the 120 kDa ß-galactosidase protein in the positive control lane [Figure 3.3.13 A]. No protein bands of the expected ~30 kDa size of recombinantly expressed CP were observed even after the transfections were repeated [Figure 3.3.13 B]. Colorimetric staining of a Western blot of the same cell lysates using rabbit raised anti-BFDV as primary antibody and anti-rabbit-AP as secondary conjugated antibody did not yield any distinctive protein bands.
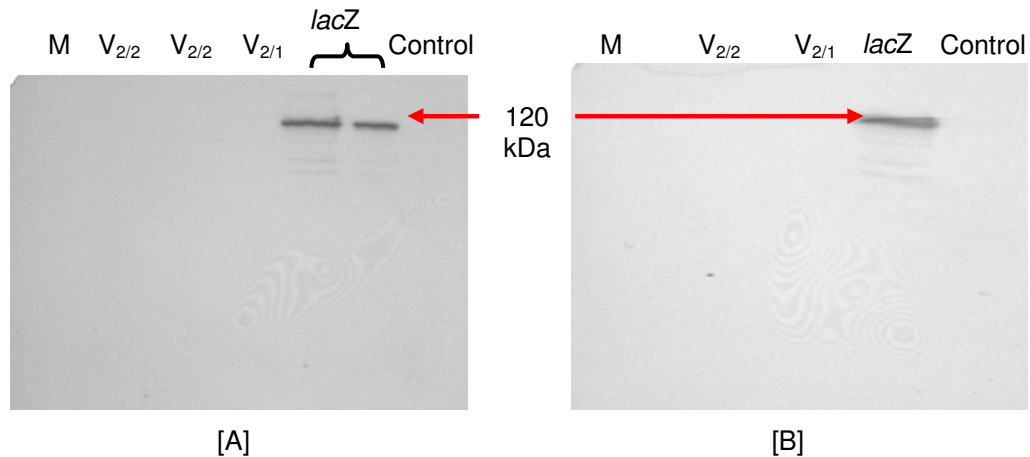
Figure 3.3.13: ß-galactosidase bands detected on a Western blot using chemiluminescent detection of the V5 epitope from samples collected from CHO cells transfected with a 6:1 ratio of FuGENE 6 Reagent:pcDNA™3.1D/V5-His/*lac*Z. Lanes $V_{2/1}$, $V_{1/2}$ and Control represent samples collected from CHO cells transfected with a 6:1 ratio of FuGENE 6 Reagent:VACC 2-1 and VACC 2-2 and untransfected cells, respectively. There were no visible bands of size ~30 kDa which is the expected size for recombinantly expressed CP. Lane M represents the PageRuler™ Prestained Protein Ladder from Fermentas.

The positive expression of ß-galactosidase from the positive control plasmid and the lack of expression of recombinant CP from transfected CHO cells gave rise to uncertainty regarding the correct orientation of the inserts in the vector pcDNA™3.1D/V5-His-TOPO® although the expected RFLP profiles for inserts in the correct orientation were observed. To further confirm the orientation and sequence of the inserts in VACC 2-1 and VACC 2-2, a single clone of VACC 2-1 and four clones of VACC 2-2 were sent to Inqaba Biotechnologies Ltd for sequencing.

3.3.6. <u>SEQUENCING RESULTS FOR VACC 2-1 AND VACC 2-2.</u>

An alignment of the two samples of plasmid DNA, VACC 2-1 and VACC 2-2 that were used in the transfection of CHO cells, indicated a discrepancy in the sequence for VACC 2-2. While the sequence for VACC 2-1 was correct, there were two bases (indicated in red) missing from the 5'-end of the reverse primer sequence of the insert (5'- AGT GCT GGG ATT GTT AGG GGC AAA CT -3') in the sequence for the plasmid VACC 2-2.

Further alignment of the four clones of VACC 2-2 indicated that only one of these clones had the entire sequence of the insert and this was not the clone that had been used in the prior transfections. The missing bases in the plasmid VACC 2-2 that was used for transfection resulted in a shift in the ORF which led to the shifting of the C-terminal 6XHis tag and V5 epitope encoded by the TOPO® vector out of frame hence the lack of detection of expressed recombinant CP.

3.3.7. SDS-PAGE AND WESTERN BLOTS OF FuGENE 6 TRANSFECTED CHO CELLS WITH VACC 2-1 AND VACC 2-2 (SECOND RUN).

CHO cells were transfected with the positive control plasmid pcDNA™3.1D/V5-His/lacZ, VACC 2-1 and the correct plasmid VACC 2-2 that contained a full insert of the CP gene in a 6:1 ratio with FuGENE 6 Reagent. Cell lysates collected 72 hrs post-transfection were separated on a Western blot and a 120 kDa band was observed in the lanes with positive control plasmid and a protein band lying between the 35 kDa and 25 kDa bands in the marker lane was observed in the lanes with VACC 2-2 [Figure 3.3.14].

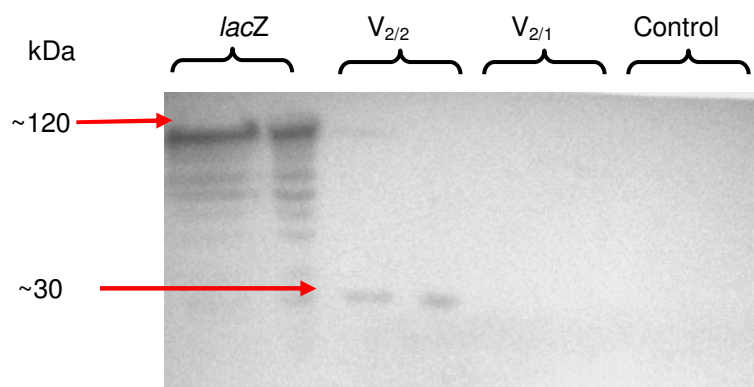**Figure 3.3.14:** A Western blot indicating the presence of recombinantly expressed ß-galactosidase bands (~120 kDa) and CP (~30 kDa) from CHO cells transfected with positive control plasmid pcDNA™3.1D/V5-His/lacZ (Lane lacZ) and VACC 2-2 (Lane $V_{2/2}$), respectively. No protein bands were observed in samples from CHO cells transfected with VACC 2-1 (Lane $V_{2/1}$) or untransfected cells (Lane Control) as expected.

The protein band in lanes with VACC 2-2 is ~30 kDa in size which is the expected size of recombinant CP. This confirms that the DNA vaccine VACC 2-2 results in expression of recombinant antigenic protein in CHO cells and is thus, functional.

The next step after confirming that the DNA vaccine is functional in mammalian cells was to validate the vaccine in an animal model. As the vaccine is to be used for the vaccination of parrots, it was decided to directly use budgerigars (*M. undulatus*) in a small vaccine trial as opposed to vaccinating mice and then moving on to the vaccination of parrots. The vaccine trial performed in this study was designed to be a very basic and preliminary indication of whether the DNA vaccine, VACC 2-2, was capable of inducing even the slightest immune response in the parrots. It was by no means intended as a conclusive indication that the vaccine is functioning at its optimum.

3.3.8. <u>RESULTS FROM THE PRELIMINARY VACCINE TRIAL USING VACC 2-2.</u>

A small-scale pilot vaccine trial was performed to test the effectiveness of VACC 2-2 in producing an immune response. Due to the nature of the vaccine trial, only 10 birds were used in total and these belonged to the species *M. undulatus* (budgerigars) which are cheap, readily available and easy to handle. The budgerigars were divided into four groups: a control group that did not receive any formulation, a group of 3 birds that were vaccinated with 50 µg of VACC 2-2 in saline and another group of 3 birds that were vaccinated with 50 µg of VACC 2-2 in Freund's incomplete adjuvant. The fourth group was made up of two budgerigars that were vaccinated with 100 µg purified recombinant CP that was expressed in baculovirus and kindly donated to us by our colleagues at UCT. The recombinant CP was administered in Freund's incomplete adjuvant as a sub-unit vaccine. All the budgerigars were vaccinated intramuscularly.

Two weeks after the first vaccination, one of the budgerigars, female number 4 who was administered VACC 2-2 in endotoxin free saline died. The budgerigar was sent in to Dr. W. Hamming at the Langenhovenpark Diere Kliniek for a post mortem report which came back stating that the bird had died from severe acute haemorrhagic bacterial enteritis that could have been stress precipitated. It was proposed that the budgerigar may already have been suffering with the disease at the time of purchase

and the additional stress of the blood collection and vaccination procedures could have contributed to its death.
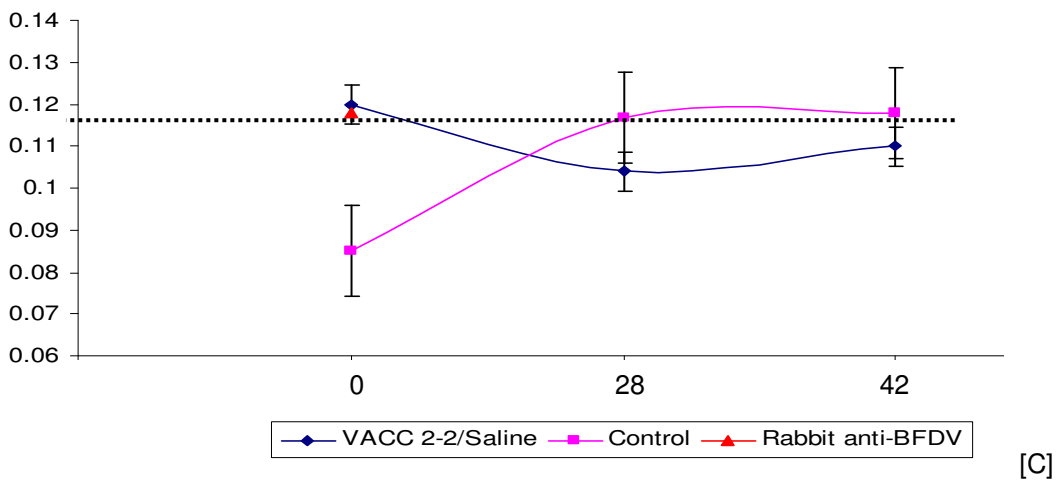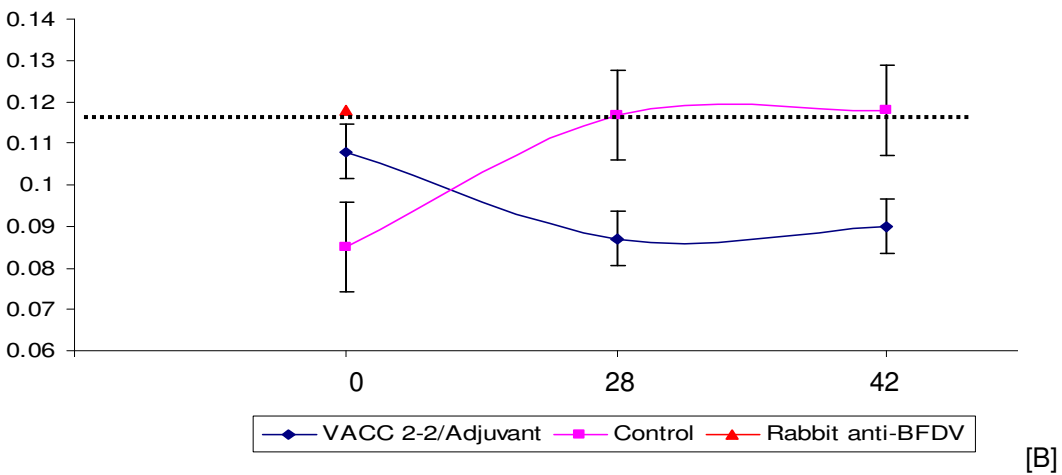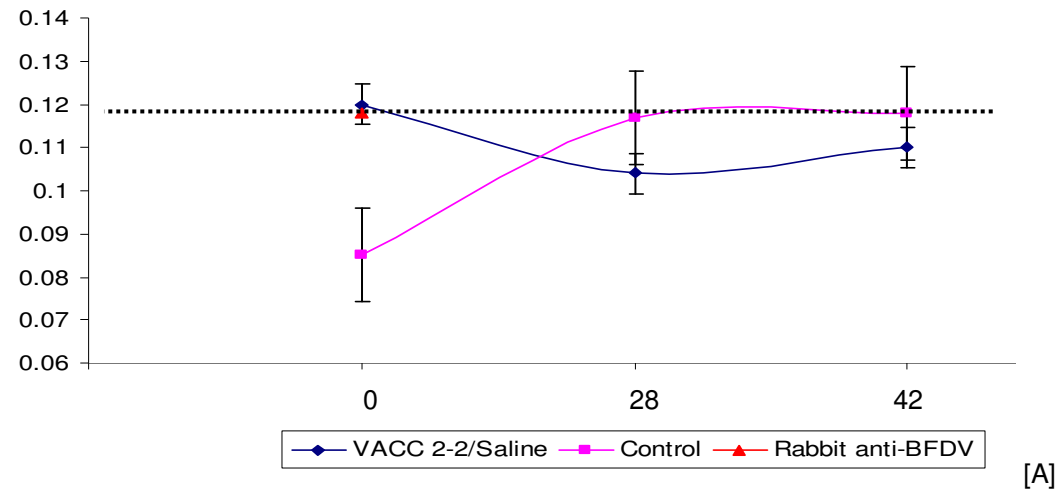

Figure 3.3.15: Graphs of average A_{450} readings obtained for groups vaccinated with VACC 2-2 in saline [A], VACC 2-2 in Freund's incomplete adjuvant [B] and purified CP in Freund's complete adjuvant [C]. The control group and rabbit anti-BFDV serum without parrot serum has been indicated in each graph.

Figure 3.3.15 A, B and C shows the results obtained for each test group that was vaccinated, using a competitive ELISA developed in the present study. The graphs were constructed using the average A_{450} readings for each group obtained at day 0, 28 and 42 post-vaccination (individual A_{450} readings for each bird are indicated in Table 3.5) and a decrease in absorbance readings was observed for each vaccinated group from day 0 to day 28. Thereafter, a slight increase in A_{450} readings is seen.

Table 3.5: Table showing the individual A_{450} readings for budgerigars (*M. undulatus*) in the preliminary vaccine trial pre- and post-vaccination.

		Days post-vaccination		
Parrot	Group	0	28	42
1	Control	0.099	0.12	0.122
2	Control	0.071	0.113	0.114
3	VACC 2-2/Saline	0.116	0.085	0.1
4	VACC 2-2/Saline	0.107		
5	VACC 2-2/Saline	0.124	0.123	0.119
6	VACC 2-2/Adjuvant	0.111	0.126	0.107
7	VACC 2-2/Adjuvant	0.096	0.071	0.055
8	VACC 2-2/Adjuvant	0.117	0.064	0.108
9	CP/Adjuvant	0.115	0.066	0.075
10	CP/Adjuvant	0.117	0.068	0.053
Rabbit anti-BFDV = 0.118, FBS = 0.121				

The dotted line in Figure 3.3.15 represents the titre reading obtained for rabbit serum without any added parrot serum and any serum that is negative for BFDV specific antibodies should be close to this reading as the concentration of parrot raised anti-BFDV is indirectly proportional to the A_{450} readings. A reduction in A_{450} readings is expected as the concentration of parrot anti-BFDV titres increase due to the inverse relationship shared by the concentration of parrot raised antibodies and formation of substrate. This means that the decrease in A_{450} readings from day 0 to day 28 for each vaccinated group is an indication of a positive antibody response (formation of BFDV specific antibodies) of the budgerigars.

The group vaccinated with the sub-unit vaccine showed the highest antibody response followed by the group vaccinated with VACC 2-2 in adjuvant then the group vaccinated with VACC 2-2 in saline. The control group shows an initial low A_{450} reading at day 0 which was unexpected but this could be due to the testing of a

serum in a single well as the volume of blood from the budgerigars was a limiting factor. In retrospect, using budgerigars (*M. undulatus*) was unsuitable for vaccine trials as they proved difficult to bleed, producing only very small volumes of blood and consequently, small volumes of serum. Unfortunately, due to this limited volume of serum which had to be further diluted 5-fold to obtain the working volume for a single well in the indirect competitive ELISA developed as part of this study, serum could only be tested once as opposed to the usual four wells that are normally employed during similar studies.

It may also be possible that this initial low A_{450} reading in the control group was due to the presence of BFDV specific antibodies. These birds were obtained from a pet shop with no history available on the birds. As PBFD affects budgerigars (*M. undulatus*) and has been demonstrated in these birds in South Africa (Albertyn *et al.*, 2004), it is possible that the birds obtained could have already had antibodies to BFDV. As can be seen from the pre-vaccination serum samples, none of the birds in the vaccinated groups showed any indication of BFDV-specific antibodies.

The large standard error observed for all the graphs indicates the wide variation seen in the A_{450} readings for each of the individual budgerigars and can also be attributed to the testing of serum in a single well. This can easily be reduced by increasing the number of birds per group in the vaccine trial as well as using larger birds to obtain more serum that in turn can be tested in multiple wells using the indirect competitive ELISA without the need for dilution. However, it is notable mentioning once more that this trial was only intended to be an indication of whether the vaccine was functional in parrots and whether it invoked any immune response which has been indicated by the reduction in A_{450} readings from day 0 to day 28 (a decrease in A_{450} readings = an increase in BFDV specific antibodies).

Immune responses have been indicated in animal model systems using "naked DNA" in saline, DNA complexed with lipids and DNA impelled either as an aerosol or by coating it onto gold beads (Hassett and Whitton, 1996). The first important consideration for a successful, protective DNA vaccine is the design of the DNA vaccine. A good DNA vaccine should be able to stimulate an appropriate cell mediated and humoral response by allowing the production of adequate amounts of antigenic protein during *in vivo* synthesis and this is verified by the production of antigenic protein in cell lines. The choice of plasmid vector plays an important role in DNA vaccine design (Garmory *et al.*, 2003) and for the purpose of this study, vector

pcDNA™3.1D/V5-His-TOPO® was successfully used to drive expression of recombinant antigenic CP in the CHO cells. The plasmid vector was chosen based on the presence of the CMV promoter and that it was used in the *Porcine circovirus type 2* (PCV-2) DNA vaccine developed by Kamstrup and co-workers (2004).

Plasmid vector pcDNA™3.1D/V5-His-TOPO® uses the CMV promoter to drive expression in eukaryotic cells. There are a number of promoters that have been used effectively in the construction of DNA vaccines such as the CMV promoter, creatine kinase promoter, SV40, ß-actin, RSV and many more (Garmory *et al.*, 2003). While these promoters have all successfully driven gene expression in various tissues, virally derived promoters have provided greater gene expression *in vivo* than other eukaryotic promoters especially the CMV promoter (Garmory *et al.*, 2003). This promoter consists of the immediate early enhancer-promoter region and has been shown to direct the highest level of transgene expression in eukaryotic tissues. An example of such expression was found in macaques injected with plasmids expressing HIV-1 Gag/Env under the regulation of the CMV promoter. This DNA vaccine showed a higher Gag- and Env-specific humoral response and T-cell proliferative responses as opposed to macaques injected with the same plasmid under the control of the endogenous AKV murine leukaemia long terminal repeat (Garmory *et al.*, 2003). However, there are some cases such as gene therapy applications using interferon-γ or tumour necrosis factor-α where CMV promoters can not be used due to the inhibition of transgene expression by these treatments. As the vector was not to be used for gene therapy but as a preventative vaccine, a vector using the CMV promoter to drive eukaryotic expression was the first choice for DNA vaccine design in the study.

PCV-2 causes post weaning multisystemic wasting syndrome (PMWS) and belongs to the same genus, *Circovirus*, as BFDV does. The DNA vaccine developed by Kamstrup *et al.* (2004) forms a practical model upon which to build a DNA vaccine candidate for BFDV because these two viruses share a number of similarities. Like BFDV, PCV-2 encodes its capsid protein from ORF 2 which has been reported to share 26% identity with that of BFDV on a nucleotide level (Crowther *et al.*, 2003). The amino acid alignment of these two genes also reveals a highly conserved sequence of 14 amino acids (YVtkLTIYVQFRqF) near the carboxyl terminus of both proteins and adjacent corresponding myristylation sites suggesting the necessity for protein function (Niagro *et al.*, 1998). If the two CPs show such a significantly important level of similarity then it would be plausible to suggest that if a DNA vaccine

constructed using the CP gene of PCV-2 by Kamstrup and co-workers (2004) was found to invoke the production of antibodies against PCV-2 in mice vaccinated using a gene gun then similarly, a DNA vaccine constructed using the CP gene of BFDV should elicit a protective immune response against BFDV in parrots vaccinated with the DNA vaccine. Although this was not conclusively established in the present study, the development of an immune response as a result of DNA vaccination with the plasmid vector pcDNA™3.1D/V5-His-TOPO® containing the CP gene of BFDV has been presented in this study.

Another important consideration when establishing a protective immune response using DNA vaccination is the route of vaccination. Immunisation of naked DNA in saline directly into the skeletal muscle has been shown to produce a higher expression of antigenic protein when compared to using DNA-lipid complexes; however, the expression of antigenic proteins from DNA-lipid complexes was enhanced when delivered intravenously (Hassett and Whitton, 1996). The initial ability of DNA vaccines to trigger humoral and cell mediated immunity was revealed with the intramuscular inoculation of mice with naked DNA in 1990 (Garmory *et al*., 2003). Since then many advances have been made in DNA vaccine technology and much has been learnt about routes of inoculation and success of different delivery systems.

Intramuscular DNA vaccines have been found to be protective in various animal models of viral diseases resulting in the production of antibodies as well as efficiently eliciting CD8^{+} CTLs capable of lysing infected cells. Antibody responses were first demonstrated for an influenza virus protein in mice and have been reported for the haemagglutinin (HA), matrix protein and nucleoprotein (NP) from influenza A (Donnelly *et al*., 1997). Other plasmid DNAs known to induce antibody responses when administered intramuscularly encoded gp120 and gp160 from HIV, gIV from bovine herpesvirus I, rabies virus surface glycoprotein, hepatitis B surface antigen and many more (Donnelly *et al*., 1997). CTL were readily demonstrated in spleen or lymph node cells from animals immunised intramuscularly with plasmid DNA encoding the NP from influenza virus, hepatitis B surface antigen, HIV *env* and much more (Donnelly *et al*., 1997). While immunisation with naked DNA has been shown to stimulate immune responses, potential disadvantages include uptake by a minor fraction of muscle cells, exposure to nucleases, use of large quantities of DNA and the need to inject into regenerating muscle in order to enhance immunity (Gregoriadis *et al*., 1997).

As little as 1 µg of HA DNA injected intramuscularly twice has been shown to completely protect mice from disease following a lethal homologous challenge (Donnelly *et al.*, 1997). Studies in rodents have demonstrated that the muscle is 100-1000 times more permissive that other tissues for the uptake and expression of DNA. Fynan *et al.* (1993) evaluated parenteral, mucosal and gene-gun routes of immunisation in both murine and avian influenza models and reported that while intramuscular and intravenous injections achieved good protection, the most efficient route of DNA vaccination was achieved using a gene gun to deliver DNA coated gold beads to the epidermis.

The unavailability of a gene gun for use in the present study eliminated this route of immunisation but considering all the positive aspects of intramuscular immunisation, the choice was made to use this route to vaccinate the birds. It would appear from the results obtained in this study that intramuscular immunisation is sufficient to stimulate an antibody response to the DNA vaccine (VACC 2-2) that was administered. However, as it can not be established that these antibody responses are protective, an aspect for further investigation would be to determine if intramuscular immunization is the best route for vaccination. It may be possible that using a gene gun to vaccinate the birds may result in higher antibody responses that will be extended for a period longer than 28 days (the length of the immune response in the present study) or that there is a protective need for the DNA to be complexed to lipids to increase antigen expression and hence increase the antibody response with regard to quantity and length of response.

The decreasing A_{450} readings from day 0 to day 28 in Figure 3.3.15 indicate that the antibody responses obtained in the present study lasted for 28 days in all the vaccinated groups and thereafter started to diminish as indicated by the slight increase in A_{450} readings up to day 42. DNA vaccines are known to result in antibody responses that last four to eight weeks upon initial vaccination but have also been reported to induce protective immune responses that last years. Intramuscular immunization of influenza HA DNA in African green monkeys resulted in durable antibody responses that lasted four months or more while in mice, immune responses lasted 20 months or more (Donnelly *et al.*, 1997). Antibody responses to a single dose of naked DNA (pRc/CMV HBS) were detectable 1-2 weeks after injection and peaked by 4-8 weeks. However, Gregoriadis and co-workers (1997) reported that a single dose of immunization with lower doses of pRc/CMV HBS under the conditions

of the study resulted in barely detectable antibody responses even by 10 weeks. Mice immunized using a gene gun responded to two vaccinations of the PCV-2 DNA vaccine developed by Kamstrup *et al.* (2004) by developing antibodies which were further boosted by a third vaccination. These antibody levels were detected after 62 days. There is a variation in the length of the immune response for different DNA vaccines in various animal models that also appears to depend on the route of inoculation, the type of antigen and the formulation of the vaccine. A combination of these factors could have possibly contributed to the length of antibody responses seen in the current study that lasted up to 42 days but appear to be decreasing from day 28.

Immune responses tend to last for a length of time that is dependent on the life cycle of the animal. Therefore, an animal with a shorter life span such as a parrot will produce an immune response for a shorter period of time as compared to a human being who has a longer life span and will produce an immune response that lasts relative to its length if life. This could also be one of the reasons for the slight increase in A_{450} readings after day 28 as budgerigars have an average life span of only five to ten years. It is plausible to speculate that if the trial had been continued for a longer period and the budgerigars boosted a few more times that the immune response would have increased further and lasted longer and is an aspect that should be considered for future investigation.

The higher immune response seen for the sub-unit vaccine is not unexpected as Blanchard and co-workers (2003) reported that protection induced by a sub-unit vaccine against PCV-2 infection was better than the one induced by a DNA vaccine against the same infection. In their study, they compared the efficacy of protection induced by a DNA vaccine (pOrf1,pOrf2 and pGM-CSF) and a sub-unit vaccine (crude lysate from recombinant baculoviruses BacOrf1 and BacOrf2) by evaluating growth parameters and clinical symptoms compared to non-vaccinated and challenged pigs. The sub-unit vaccine significantly reduced the duration of the pyrexic phase during the third week post-infection while the DNA vaccine did not have any effect on the pyrexic phase. They also reported earlier seroconversion within 2 weeks after the first injection of the sub-unit vaccine while the DNA vaccine only elicited seroconversion after challenge which was administered 11 days after the second injection. However, higher antibody titres were observed for the DNA vaccinated group after challenge while that of the sub-unit vaccinated group remained lower, suggesting the absence of PCV-2 replication and boosting effect after challenge.

DNA vaccination was also able to prime the antibody response since an earlier seroconversion was observed in the DNA vaccinated group after challenge.

It can be suggested that there is a possibility that the antibody responses in the group of budgerigars vaccinated with VACC 2-2 in saline and adjuvant may have increased if the present trial study had been extended to include a challenge experiment. If the immune response to DNA vaccination for BFDV infection were to follow a similar pattern to that observed by Blanchard *et al.* (2003) for PCV-2 infection (as BFDV and PCV-2 are significantly similar), then it may be possible that the DNA vaccine (VACC 2-2) will elicit rapid production of BFDV specific antibodies in higher titres once the budgerigars are challenged. A challenge experiment would also allow for the determination of whether the DNA or sub-unit vaccines or both are capable of inducing protective immune responses by monitoring clinical symptoms of the challenged birds.

The length and intensity of the immune response is dependent on many variable factors and therefore it is important for the vaccine trial to be expanded and include challenge studies so as to establish the optimum parameters for vaccination to achieve a protective immune response. While the present study does not provide proof of a protective response, it can be suggested that both the DNA vaccine, VACC 2-2, and the sub-unit vaccine have invoked the production of antibodies in the vaccinated budgerigars. Although a budgerigar died during the trial, the cause of its death was attributed to a bacterial infection that was stress precipitated as indicated on the post mortem report and not due to the DNA vaccine itself. It has therefore also been preliminarily established that the DNA vaccine and sub-unit vaccine are both safe to use in parrots as no adverse reactions were observed at the site of inoculation or during the period of the vaccine trial.

3.4. CONCLUSIONS.

In conclusion, a potential DNA vaccine candidate (VACC 2-2) has been developed that shows expression of recombinant antigenic CP in transiently transfected CHO cells and invokes weak antibody responses in parrots from the species, *M. undulatus* (budgerigars) when administered in saline as well as in Freund's incomplete adjuvant. The antibody response invoked by the DNA vaccine candidate is lower than that produced in response to purified recombinant CP administered as a sub-unit vaccine.

However, the present study does not provide proof that these antibody responses are protective but it can be suggested that both the DNA vaccine candidate and sub-unit vaccine are safe to be administered to parrots. The safety and efficacy of DNA vaccines has been successfully shown in animal models against viral, bacterial, protozoan and fungal antigens indicating the potential utility of these vaccines as promising antigen delivery systems and may find application in the prevention of PBFD.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS.

PBFD is a readily recognisable disease of wild and captive psittacine species worldwide and is characterised by the progressive, symmetrical loss of feathers and presence of abnormal feathers. It is known to affect more than 40 species of Old and New World psittaciformes worldwide (Wylie & Pass, 1987; Sanada *et al.*, 1999; Kiatipattanasakul-Banlunara *et al.*, 2002; Rahaus and Wolff, 2003; Ritchie *et al.*, 2003; Albertyn *et al.*, 2004; Heath *et al.*, 2004; Hsu *et al.*, 2006). The disease is widespread in South Africa where bird breeders lose at least 10% of their psittacine breeding stocks to PBFD a year. An estimate of the value of breeding stocks lost brings the amount to approximately R 4 000 000 a year. PBFD is also a major threat to a number of parrot species and in South Africa, the already endangered Cape parrot (*Poicephalus robustus*) and the vulnerable black-cheeked lovebird (*Agapornis nigrigenis*) are facing extinction due to PBFD outbreaks.

The causative agent of PBFD is BFDV, a highly resilient and environmentally stable virus that is not easily eliminated using disinfection. Good biosecurity measures can be effective in controlling the spread of infection but they are not a preventative measure. PBFD is a fatal disease especially in young birds aged below 3 years and once a bird becomes infected by the virus it is most likely to succumb to secondary infections as a result of immunosuppression caused by BFDV attack on the developing bursa. Older birds that become infected usually become carriers of the virus and a source for vertical transmission of BFDV. It is therefore vital to develop a preventative measure in the form of a safe yet effective vaccination program.

Numerous attempts to culture BFDV in tissue/cell culture and in embryonated eggs have been unsuccessful which has largely contributed to the lack of reliable diagnostic tests for accurate diagnosis of infected birds as well as the development of a vaccine. An inactivated vaccine developed by Ritchie and co-workers (1992) and Raidal *et al.* (1993b) using purified BFDV particles inactivated with ß-propiolactone was unsuitable for commercial production as it requires purification of virus from infected tissues which is time-consuming, expensive and an ethically questionable process. It also results in low yield of viral particles unsuitable for large-scale

production and quite often these purification procedures results in the contamination of the antigen with host proteins that cross react in serological tests.

The drawbacks involved in the production of an inactivated vaccine for the prevention of PBFD can be compensated for by new vaccine development strategies being used today, specifically sub-unit and DNA vaccines. These vaccines make use of recombinant DNA technology which enables only the antigen required for protective immunization to be presented to the host's immune system making the protective response more specific and safe. While sub-unit vaccines are an attractive method of vaccination in humans and animals, these vaccines are difficult to prepare due to the purification procedures involved and are therefore expensive. DNA vaccines overcome this difficulty in preparation and high cost of production because the immunizing protein is expressed within the host cells where it is presented to the host's immune system bypassing the need for expensive and difficult protein purification procedures.

DNA vaccines are very stable and unlike most conventional vaccines do not require the cold chain. This is of particular importance in delivering vaccines in veterinary fields and in remote areas of developing countries (Shams, 2005). These vaccines eliminate the risk associated with live vectors such as infection, latency and immunity against the vector and the expressed antigens readily go through processing and presentation to the immune system to induce cell mediated and humoral responses. However, DNA vaccines also have some limitations especially as they can only induce protection against proteins but not polysaccharides so they are not yet an alternative for polysaccharide-based vaccines (Shams, 2005). There is no extensive homology between mammalian cells and plasmid DNA so it is unlikely that homologous recombination will occur but the possibility of random DNA integration into the host genome exists. Though there are currently no reports on the adverse effects of DNA vaccines, it is important to be aware of the possibilities especially when registering these vaccines for human or animal use.

As the advantages of DNA vaccines outweigh their limitations, the primary aim of the present study was to develop a potential DNA vaccine candidate that could be used to protect parrots against PBFD. Research and diagnosis of PBFD is highly limited due to the inability to propagate the virus *in vitro* which means that there is currently no serological test available in the form of an ELISA to monitor immune responses in parrots. Birds are diagnosed using PCR and HA and HI assays but there is no "gold

standard" for the serological detection of BFDV (Johne *et al.*, 2004). This is because the HA and HI assays have serious problems in standardization and interpretation of results due to varying results obtained using erythrocytes collected from different species as well as individuals of the same species (Sanada and Sanada, 2000).

It was therefore important to establish a serological test, an ELISA, in the present study that would be able to detect antibody responses in parrots when testing the efficacy of the potential DNA vaccine candidate. The only tool available for establishing an ELISA during the study was polyclonal antibodies raised in rabbits injected with purified BFDV particles. Due to the lack of a readily available source of wild type virus for use as coating antigen an alternative source of antigenic protein was required and so recombinant DNA technology was used to produce CP which has been suggested to be the epitopic protein of BFDV.

Two expression systems were evaluated for the production of recombinant CP: a bacterial and eukaryotic (yeast) expression system. These two systems were studied to determine which system yielded higher concentrations of recombinant CP that was applicable in the ELISA as bacterial systems do not modify proteins post-translationally while yeast systems do. The need for recombinant CP to be post-translationally modified for the detection of BFDV specific antibodies in parrot sera using an ELISA was not known. The pBAD bacterial expression system was successful in producing recombinant CP while the yeast system was not. Failure of the yeast system to produce recombinant antigen could be attributed to a complete lack of expression from the TEF promoter of the yeast vector pKOV136 or the expression of very low concentrations of recombinant CP that were undetectable using SDS-PAGE, Western blot and ELISA. The yeast vector pKOV136 is a single-copy expression vector and reports on studies of production of recombinant protein using single-copy yeast vectors compared to multi-copy expression vectors indicate that much higher concentrations are obtained with multi-copy vectors (Nicaud *et al.*, 2002).

The recombinant CP expressed from the pBAD/His vector was successfully applied in both an indirect ELISA and an indirect competitive ELISA and used to detect the polyclonal rabbit raised antibodies against whole virus as well as BFDV specific antibodies raised in parrots that were naturally infected. Previous reports by Johne and co-workers (2004) and Shearer *et al.* (2008) indicate the successful use of bacterially expressed and baculovirus expressed recombinant CP, respectively, in

ELISAs to detect BFDV specific antibodies in naturally infected parrots and immune responses in mice vaccinated with a sub-unit vaccine consisting of purified baculovirus expressed CP in Freund's incomplete adjuvant. The ELISAs established in the present study were functional although the coating antigen used was non-purified or partially purified. However, if the competitive ELISA is to be used as a reliable diagnostic test, future work should include optimising the purification of recombinant CP to homogeneity to increase sensitivity and specificity and standardizing the ELISA to allow accurate interpretation of results. Like the HA and HI assays, standardization of the ELISA faces a number of limitations which include the need for large sampling numbers of known positive and negative samples and a reliable diagnostic test to accurately distinguish positive and negative birds. At present neither the PCR nor the HA and HI assays are 100% reliable.

With a competitive ELISA in place to detect antibody responses in parrots, it was possible to carry out a preliminary trial to determine if the potential DNA vaccine candidate developed during the study was suitable for further investigation. The DNA vaccine candidate consisted of the CP gene contained in the plasmid vector pcDNA™3.1D/V5-His-TOPO® (Invitrogen) and was shown to express recombinant CP when transiently transfected into CHO cells. This was a good indication that the vaccine candidate was functional and would facilitate the *in vivo* synthesis of antigenic CP when injected into parrots. Although the design of the vaccine trial carries a number of flaws due to limiting factors such as the species of birds used (*Melopsittacus undulatus* - budgerigars) as well as the number of birds per vaccinated group, it is important to note that the purpose of the trial was to detect any antibody response in vaccinated parrots so as to determine if the potential DNA vaccine candidate merited further investigation.

The trial preliminarily indicates the ability of parrots vaccinated with the DNA vaccine or a sub-unit vaccine to form antibodies in response to either of the vaccines. Parrots vaccinated with the sub-unit vaccine had higher antibody titres than parrots vaccinated with the DNA vaccine. This is not surprising as a study by Blanchard and co-workers (2003) reported better protection against PCV-2 when pigs were vaccinated with a sub-unit vaccine consisting of purified capsid protein as compared to a DNA vaccine containing the *Orf2* gene which encodes the capsid protein. The capsid protein of PCV-2 is significantly similar to the CP of BFDV as it shares 26% identity in nucleotide sequence and similarities in the amino acid sequences. Both viruses are also classified in the genus *Circovirus*.

As the competitive ELISA has not been standardized and because the results are statistically incorrect due to the testing of serum in a single well (as a result of small blood volumes), this study can not conclude that these antibody responses are protective and significantly accurate. However, it can be suggested that the antibody responses obtained in the trial show promise for further investigation into the use of the DNA vaccine candidate and the sub-unit vaccine as suitable programs for prevention of PBFD. The first step would be to expand the vaccine trial using a bigger species of parrot so that adequate volumes of blood and consequently serum can be obtained. The sera should be tested in at least four wells per sample and used in the undiluted form. A larger number of birds per vaccinated group should also be used as large sample numbers are required for statistical validation of any assay. The increased number of birds and multiple testing of sera will reduce the large standard errors obtained in the present trial and reflect the ability of either vaccine to induce antibody responses more accurately.

An interesting aspect to look into further would be to purify the bacterially expressed recombinant CP to homogeneity and use it as a sub-unit vaccine to vaccinate parrots and compare the antibody responses to those obtained using the sub-unit vaccine consisting of baculovirus expressed recombinant CP. Bacterially expressed recombinant proteins are not modified post-translationally and it would be of interest to know if post-translational modification is required for the recombinant CP to invoke an immune response and if not, is the immune response comparable to that achieved with post-translationally modified CP.

Another aspect that needs to be investigated where the DNA vaccine is concerned is to determine a way of increasing the antibody response either by various modes of delivery or by increasing the potency using adjuvants or immunostimulatory molecules either as recombinant proteins or as co-transfected genes. The route of immunisation has been found to be an important determinant of immunogenicity where Fynan *et al.* (1993) investigated protective immunizations by parenteral, mucosal and gene-gun inoculations and reported that while intramuscular and intravenous injections achieve good protection, the most efficient method of DNA immunization was achieved using a gene gun to deliver DNA-coated gold beads to the epidermis. Delivery of DNA by the gene gun requires much less DNA ($^1/_{250} - ^1/_{500}$) to achieve protection than that required for intramuscular injection and the DNA is delivered directly to the epidermis where cells such as Langerhans cells present

antigen to the immune system. This system of delivery results in highly efficient transfection and antigen presentation.

DNA itself has been shown to have adjuvant properties through CpG motifs and has been used to modulate DNA vaccines by linking the motifs to the relevant antigen (Klinman *et al.*, 1999). Cytokine proteins have also been used to augment immune responses and a number of studies have shown the use of cytokine, chemokine and co-stimulatory genes as genetic adjuvants for DNA vaccines (Scheerlinck, 2001). The GM-CSF gene has been one of the most studied genetic adjuvants and has a good track record of enhancing humoral responses which is dependent on the time at which the plasmid encoding cytokine is injected relative to the time of injection of the DNA vaccine (Scheerlinck, 2001). However, a true reflection of the functionality of the DNA vaccine can only be obtained during challenge studies as measurement of immune parameters does not substitute challenge trials for evaluating DNA vaccination. It is therefore, imperative that once a reliable diagnostic test is developed, that the DNA and sub-unit vaccines are used in a challenge trial that will monitor symptomatic changes in the physical appearance of challenged birds as well as their immune responses.

Among the objectives of vaccinology in the veterinary field is the intent to provide cost effective approaches to prevent and control infectious diseases and to improve the welfare of animals. Although traditional vaccinology has come a long way since the discovery of the smallpox vaccine by Edward Jenner, pathogens such as viruses, bacteria and protozoa amongst others are constantly evolving to adapt to a changing environment ensuring their survival by even the smallest changes to their genomes. The emergence of stronger and more resistant pathogens, new microbial strains with the ability to evade traditional vaccines and the threat of bioterrorism is an indication that scientists have to develop new technologies for fighting infectious diseases. Gene based vaccines such as DNA vaccines have emerged as promising alternatives to traditional vaccines and though there is still a need for improvement, these vaccines together with recombinant DNA technology will result in a platform technology for a variety of diseases, one of which may be PBFD.

SUMMARY.

Psittacine beak and feather disease (PBFD) is a readily recognisable dermatologic condition in wild and captive psittacines worldwide. It is caused by *Beak and feather disease virus* (BFDV) which is classified in the family *Circoviridae* and the genus Circovirus. BFDV has a circular ss-DNA genome consisting of seven open reading frames (ORFs), three being conserved in all BFDV isolates, ORF 1 which encodes the Rep protein, ORF 2 which encodes the coat or capsid protein (CP) and ORF 5 which encodes a protein whose function is as yet unknown. General symptoms of the disease include the symmetrical loss of feathers, feather abnormalities, beak and claw deformities, weight loss, anorexia and immunosuppression. The inability to grow BFDV in tissue culture or in embryonated eggs has hindered the routine diagnosis of PBFD affected birds and the development of reliable diagnostic tests and an effective vaccination program.

PBFD is widespread in South Africa, leading to a loss of at least 10% of psittacine breeding stocks annually. The disease is also a major threat to the already endangered Cape Parrot (*Poicephalus robustus*) and the black-cheeked lovebird (*Agapornis nigrigenis*) and it is only a matter of time before we may see the extinction of these and other parrot species due to the lack of a preventative vaccine. The economical and natural implications of the attack by PBFD led to the aims of the present study which were to develop a potential DNA vaccine candidate, develop an expression system for production of recombinant CP as antigenic protein and establish an enzyme linked immunosorbent assay for the detection of BFDV-specific antibodies in parrots.

The entire CP gene which has been suggested to encode for the epitopic protein of the virus was amplified by polymerase chain reaction (PCR) and ligated into a bacterial vector, pBAD/His B or a yeast vector, pKOV136 for expression of recombinant CP in *Escherichia coli* or *Yarrowia lipolytica*, respectively. Alternatively, CP gene PCR products were ligated into the mammalian expression vector pcDNA™3.1D/V5-His-TOPO® which was the vector of choice for DNA vaccine design and used to transiently transfect Chinese hamster ovary cells. Subsequently, the candidate DNA vaccine was used in a basic vaccine trial where budgerigars (*Melopsittacus undulatus*) were vaccinated either with the DNA vaccine candidate or a sub-unit vaccine consisting of purified recombinant CP. Expression of recombinant

CP was monitored using polyacrylamide gel electrophoresis (PAGE), chemiluminescent and colorimetric detection on Western blots and ELISAs.

While expression of the recombinant CP was unsuccessful in the yeast system using pKOV136, expression of recombinant CP was achieved in *E. coli* cells using the pBAD vector. Recombinant CP was partially purified and applied in both indirect and indirect competitive ELISAs as coating antigen for the detection of BFDV specific antibodies. Using the established ELISAs, BFDV specific antibodies could be detected in naturally infected parrots as well as in budgerigars vaccinated with the DNA vaccine and sub-unit vaccine. Comparable results were obtained when non-purified recombinant CP was applied in the ELISAs in lieu of partially purified recombinant CP.

Vaccinated budgerigars formed BFDV specific antibodies in response to the DNA vaccine and sub-unit vaccine that were detected using the indirect competitive ELISA established in the study. The antibody responses to the sub-unit vaccine were higher than those in response to vaccination with the DNA vaccine candidate. Although the indirect competitive ELISA could not provide an indication of whether these antibody responses are protective, the results obtained during the trial are a preliminary indication that both the DNA vaccine and sub-unit vaccine may be functional in parrots and safe to use as no adverse reactions were observed.

OPSOMMING.

Psittacine beak and feather disease (PBFD) is 'n maklike herkenbare dermatologiese toestand in wilde en gevange papegaaie wêreldwyd. Hierdie siekte word veroorsaak deur die *Beak and feather disease virus* (BFDV) wat in die familie *Circoviridae* en die genus Circovirus geklassifiseer word. BFDV het sirkulêre enkelstring DNA genoom wat uit 7 oopleesrame (OLRe) bestaan, 3 van hierdie OLRe is gekonserveerd in alle BFDV isolate. OLR 1 kodeer vir die Rep proteïen, OLR 2 vir die kapsied proteïen (KP) en OLR 5 kodeer proteïen waarvan die funksie onbekend is. Algemene simptome van die siekte sluit in: die simmetriese verlies van vere, veerabnormaliteite, bek en klou abnormaliteite, gewigsverlies, anorexia en immuunonderdrukking. Die onvermoë om BFDV in weefselkulture of geëmbrioneerde eiers te kweek, vermoeilik die roetine diagnose van PBFD geaffekteerde voëls en die ontwikkeling van betroubare diagnostiese toetse sowel as effektiewe inentingsprogram.

PBFD is wydverspreid in Suid Afrika en lei tot die verlies van ten minste 10% van papegaai broeivoëls jaarliks. Die siekte is ook groot bedreiging vir die alreeds bedreigde Kapse papegaai (*Poicephalus robustus*) en die black-cheeked lovebird (*Agapornis nigrigenis*), dit is slegs 'n kwessie van tyd voordat die totale uitwissing van hierdie en ander papagaai spesies waargeneem word weens die afwesigheid van 'n voorkomende inentingsprogram. Die ekonomiese en natuurlike implikasies van PBFD het gelei tot die uitkomstes van die huidige studie, wat ingesluit het: die ontwikkeling van 'n potensiële DNS entstof kandidaat, om 'n uitdrukkingssisteem te ontwikkel vir die produksie van rekombinante KP as antigeniese proteïen sowel as om 'n enzyme linked immunosorbent assay (ELISA) daar te stel vir die waarneming van BFDV-spesifieke teenliggame in papagaaie.

Die volledige KP geen, wat voorgestel is om die epitopiese proteïen van die virus te wees, was geamplifiseer deur polimerase ketting reaksie (PKR) en geligeer in die bakteriese vektor, pBAD/HisB of in die gis vektor, pKOV136 vir uitdrukking van die rekombinante KP in *Escherichia coli* of *Yarrowia lipolytica*, onderskeidelik. Alternatiewelik, was die PKR produkte van die KP geen geligeer in die soogdier uitdrukkingsvektor pcDNA™3.1D/V5-His-TOPO® wat die vektor van keuse was vir die ontwikkeling van die DNS entstof, dit was gebruik om Chinese hamster ovarium selle te transfekteer. Die kandidaat entstof was gebruik in 'n basiese entings proef waar budgerigars (*Melopsittacus undulatus*) geënt was met of die DNS entstof of 'n sub-

eenheid entstof wat bestaan het uit gesuiwerde rekombinante KP. Uitdrukking van die rekombinante KP was gemonitor deur poli-akrielamied gel elektroforese (PAGE), chemi-illuminensie en kalorimetriese waarneming op Western blots en ELISAs.

Terwyl uitdrukking van die rekombinante KP onsuksesvol in die gis vektor sisteem met die gebruik van pKOV136 was, was uitdrukking van die rekombinante KP bereik in *E. coli* selle met gebruik van die pBAD vektor. Rekombinante KP was gedeeltelik gesuiwer en toegepas in beide die indirekte en die indirekte kompetitiewe ELISAs as bedekkings-antigeen vir die bepaling van BFDV spesiefieke teenliggame. Deur die daargestelde ELISAs te gebruik kon BFDV spesifieke teenliggame waargeneem word in natuurlik geïnfekteerde papagaaie sowel as in budgerigars wat met die DNS en sub-eenheid entstof geënt was. Vergelykbare resultate was verkry met gebruik van die nie-gesuiwerde KP in die ELISAs en die gedeeltelik-gesuiwerde rekombinante KP.

Geënte budgerigars het BFDV spesifieke teenliggame geproduseer in reaksie op die DNS entstof en die sub-eenheid entstof, die teenliggame was waargeneem met gebruik van die indirekte kompetitiewe ELISAs wat in hierdie studie daargestel was. Die teenliggaam reaksies op die sub-eenheid entstof was hoër as die reaksie met gebruik van die DNS entstof kandidaat. Alhoewel die indirekte kompetitiewe ELISA nie 'n indikasie kon verskaf of hierdie teenliggaam reaksies beskermend van aard was nie, is die resultate wat verkry is gedurende die proef 'n voorlopige indikasie dat beide die DNS entstof en die sub-eenheid enstof moontlik funksioneel in papagaaie kan wees en dat dit veilig is om te gebruik siende dat geen newe effekte waargeneem was nie.

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