IDENTIFICATION OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) RECEPTORS THROUGH THE USE OF RECOMBINANT CAPSID PROTEIN, VP2

by

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PREFACE

The experimental work described in this dissertation was carried out in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg campus), from January 2011 to December 2014, under the supervision of Dr PR Vukea and co-supervision of Prof. Theresa H. T. Coetzer.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

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DECLARATION – PLAGIARISM

I, Kayleen Fransiena Brien declare that

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ABSTRACT

Infectious bursal disease virus (IBDV) is a non-enveloped Birnavirus which infects the immature antibody producing B-cells of the bursa of Fabricius in young chickens. The virus causes infectious bursal disease (IBD) which is highly contagious and immunosuppressive. A compromised immune system in infected chickens leaves them susceptible to other opportunistic pathogens and as a result increases their mortality rate. Major economic losses in the commercial poultry industry are subsequently experienced in affected regions. Currently vaccines are used to control IBDV infection, however, their efficacy is affected by factors such as the presence of maternally derived antibodies in young chickens which reduces vaccine load, the continuous emergence of new virulent IBDV strains and bursal atrophy caused by some vaccines. It is therefore important to consider new ways of controlling the virus such as targeting specific stages in the virus life cycle. Since virus attachment to host cell receptor(s) is the most crucial step in the virus life cycle, developing novel antiviral agents which prevent viral entry represents a good alternative strategy for IBDV control. Identification of receptor binding proteins and receptors of host cell membranes is required for antiviral development. The receptor binding protein and outer capsid of IBDV is VP2, however, the receptor(s) utilised by IBDV to gain entry into host cells have not been conclusively identified.

Recombinant VP2 was used to identify possible IBDV receptor(s) on bursal plasma membranes using a virus overlay protein binding assay (VOPBA) and affinity chromatography. Therefore, VP2 was heterologously expressed in an *Escherichia coli* and a *Pichia pastoris* expression system as a 64 kDa fusion protein and a 47 kDa protein respectively. In addition, both systems expressed VP2 as high molecular mass proteins which were confirmed by electro-elution and western blotting. Although purification of VP2 expressed in the *E. coli* system was a challenge because VP2 expressed as inclusion bodies, polyclonal chicken anti-VP2 antibodies were produced using VP2 expressed in this system. Purification of VP2 expressed in *P. pastoris* was easier and produced a greater yield of VP2 which was used to produce a VP2-coupled affinity matrix for the purification of chicken anti-VP2 antibodies and for the purification of VP2-binding proteins of the bursal plasma membrane. Moreover, peptides were selected from the VP2 amino acid sequence and use to raise polyclonal chicken anti-VP2 peptide antibodies for comparative

identification against chicken anti-VP2 antibodies of possible IBDV receptor(s). Two IBDV VP2-binding proteins with molecular masses of 70 and 32 kDa of the bursal plasma membrane were identified in a VOPBA using recombinant VP2 or IBDV and chicken anti-VP2 antibodies. In addition to the VOPBA, four IBDV VP2-binding proteins with molecular masses of 70, 60, 45 and 32 kDa were affinity purified on a VP2-coupled affinity matrix. Analysis of the affinity purified proteins by mass spectrometry identified five proteins which share common peptides which include, the Ig-gamma chain and Ig-lambda chain of *Gallus gallus*, outer major protein of *Serratia marcescens*, the 60 kDa chaperonin of *Pseudomonas fluorescens* and elongation factor-Tu of *Yersinia pestis.* The results strongly suggest that an Ig-receptor like protein may form part of the IBDV receptor, however, much further work is required in order to establish whether the chicken homologues of the identified bacterial sequences are part of the putative bursal receptor. It is believed that the bacterial proteins contain common peptides with chicken proteins of the chicken genome which has not been fully annotated as yet. Taken together, this study successfully used VP2 to identify possible IBDV receptor(s) on bursal plasma membranes which could ultimately lead to the development of antiviral agents targeted at IBDV entry.

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LIST OF ABBREVIATIONS

2×YT	2 × yeast tryptone medium
2×YT-Amp	yeast tryptone media containing ampicillin
BCA	bicinchoninic acid
BMGY	buffered media glycerol yeast extract
BMM	buffered minimal media
BLAST	basic local alignment search tool
BSA	bovine serum albumin
BSNV	blotched snakehead virus
CEF	chicken embryo fibroblast
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
DXV	drosophila X virus
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
G	relative centrifugal force
GST	glutathione S transferase
HIV	Human immunodeficiency virus
HRPO	horse radish peroxidise
HVT	herpesvirus of turkey
IBD	infectious bursal disease
IBDV	infectious bursal disease virus

IgG	immunoglobulin G
IgY	immunoglobulin Y
IPNV	infectious pancreatic necrosis virus
IPTG	isopropyl-beta-D-thiogalactopyranoside
LSCC-BK3	a bursal-derived lymphoblastoid cell line
MBS	*m*-maleimidobenzoyl-N-hydroxysuccinimide ester
kDa	kilodalton
MEC	molecular exclusion chromatography
min	minute
Mr	relative molecular mass
OD600	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PVDF	polymer of vinylidene fluoride
RT	room temperature
RT-PCR	real-time polymerase chain reaction
SAP	shrimp alkaline phosphatise
SDS	sodium dodecyl sulphate
sIg	surface immunoglobulin
SVP	subviral particle
TAE	tris acetate buffer
TBS	tris buffered saline
TEMED	N,N,N’,N’-tetramethyl ethylene diamine
TPP	three phase partitioning

Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
Trx	thioredoxin
TV-1	Tellina virus-1
Ve	elution volume
VLP	virus-like particle
Vo	void volume
VOPBA	virus-overlay protein binding assay
VP	viral protein
Vt	total column volume
vvIBDV	very virulent IBDV strains
YNB	yeast nitrogen base
YP	yeast extract, peptone
YPD	yeast peptone dextrose media

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Infectious bursal disease virus (IBDV) is a Birnavirus which mostly affects young chickens, causing infectious bursal disease (IBD) (Kibenge *et al.*, 1988). The disease is highly contagious and was first discovered in 1960 in a town called Gumboro in Delaware, USA (Cosgrove, 1962) and is therefore referred to as Gumboro disease (Muller *et al.*, 2003). IBD has had a significant economic impact on the poultry industry (Muller *et al.*, 2003, Lee *et al.*, 2006a) and has spread to many countries in the world, including South Africa (Horner *et al.*, 1994, Vukea *et al.*, 2014).

The virus infects immature B-cells in the bursa of Fabricius of young chickens and replicates in the cytoplasm of infected cells (Kibenge *et al.*, 1988, van den Berg *et al.*, 2000, Muller *et al.*, 2003). This leads to the depletion of the B-lymphocytes, which in turn results in an immunosuppression. Clinical signs of the disease are observed in chickens between 3-6 weeks old (Muller *et al.*, 2003) and include ruffled feathers, watery diarrhoea, trembling, and severe prostration (Cosgrove, 1962). Sub-clinical effects include immunosuppression, which leaves chickens susceptible to other pathogens and mortality levels increase as a result of increased frequency of external infections (Saif, 1991).

The current control strategy for IBDV is vaccination (Muller *et al.*, 2012). Multiple vaccines are currently available, but the vaccine effectiveness is often reduced due to maternally acquired antibodies (Corley and Giambrone, 2002). These antibodies have been shown to neutralise IBDV vaccines and therefore decrease the vaccine virus load, which is required to achieve immunity (Corley *et al.*, 2002, van den Berg *et al.*, 2004, Zhou *et al.*, 2010). Vaccines have been shown to cause moderate bursal atrophy (Snyder, 1990, Zhou *et al.*, 2010) such as Nobilis® Gumboro inac (Intervet International B.V., Egypt), an inactive IBDV vaccine. Therefore, there is still a need to explore other methods to control or prevent the disease.

Viral infections can be controlled through anti-viral agents, which target crucial processes in the viral life cycle. One such process for IBDV is viral entry into host cells during infection. Such drugs have been developed for HIV entry into the host

cell (Kilby and Eron, 2003). Due to the existence of new variant strains of HIV, targeting viral entry is a good approach to prevent infection (Kilby and Eron, 2003). Success has been achieved in HIV-receptor drug development and one of the drug compounds, PRO 542, was found to be “well tolerated” and its antiviral activity was reported in both adults (Jacobson *et al.*, 2000) and children (Shearer *et al.*, 2000). One of the first steps in understanding viral entry is identification of host receptor proteins utilised.

Bursal membrane receptor proteins which IBDV uses to gain entry have not been conclusively identified. Thus far, chicken heat shock protein 90 (cHsp90) has been identified as a constituent of the bursal membrane receptor through the use of whole virus and subviral particles (SVP) in different cell lines (Lin *et al.*, 2007, Zhu *et al.*, 2008). The aim of the present study was to use the outer capsid protein, VP2 which has also been shown to be the receptor binding protein (Yip *et al.*, 2007) to identify the receptors on the bursal membranes.

1.2 INFECTIOUS BURSAL DISEASE VIRUS

1.2.1 Taxonomy and Structure

IBDV belongs to the *Avibirnavirus* genus of the Birnaviridae family which consists of icosahedral non-enveloped viruses (Dobos *et al.*, 1979). The family includes infectious pancreatic necrosis virus (IPNV) of young salmonid fish (Cohen *et al.*, 1973), Drosophila X virus (DXV) of *Culicoides spp.* in the genus *Entomobirnavirus* (Dobos *et al.*, 1979, Teninges *et al.*, 1979), blotched snakehead virus (BSNV) (Da Costa *et al.*, 2003), Tellina-1 virus (TV-1) (Nobiron *et al.*, 2008) and oyster virus (OV) of bivalve molluscs in the genus *Aquabirnavirus* and the Espirito Santo virus (ESV) (Vancini *et al.*, 2012). The IBDV icosahedron has a single-shelled capsid and is about 60 nm in diameter (Dobos *et al.*, 1979, Bottcher *et al.*, 1997). The icosohedral structure is based on a T=13 lattice (Figure 1.1A and B) with the outer surface of the capsid formed by trimeric subunits of VP2 (Bottcher *et al.*, 1997, Coulibaly *et al.*, 2005).

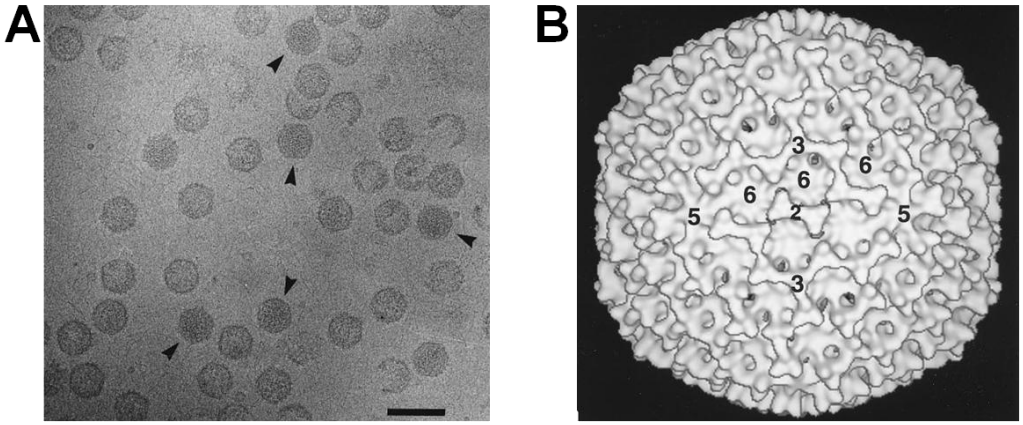

Figure 1.1 The IBDV virion. (A) Electron micrograph of IBDV particles in an unstained, frozen, hydrated preparation. Arrowheads point to IBDV particles. Bar, 100 nm **(B)** Three-dimensional structure of a frozen hydrated IBDV particle at 2 nm resolution. The two, three, five and six fold axes are marked to indicate the T=13 architecture, (Bottcher et al., 1997).

The birnavirus genome is made up of two segments of dsRNA, segment A and segment B. Segment A is 3.4 kb in size and has two open reading frames (ORFs), A1 and A2, which partially overlap (Figure 1.2). ORF A1 encodes VP5, a 17 kDa non-structural protein that assists in the release of the IBDV progeny (Lombardo *et al.*, 2000). VP5 is a cysteine rich class 2 membrane protein which has a cytoplasmic N-terminus and an extracellular C-terminal domain and accumulates in the cytoplasm until it allows for the release of the virus (Lombardo *et al.*, 2000). The larger ORF A2 codes for a 110 kDa polyprotein (NH_2-pVP2-VP4-VP3-COOH) which is proteolytically cleaved by VP4 to yield precursor VP2 (pVP2) (Met^{1}-Ala^{512}; 54 kDa), mature VP4 (Ala^{513}-Ala^{755}, 28 kDa) and VP3 (Ala^{756}-Glu^{1012}, 32 kDa) (Muller and Becht, 1982) (Figure 1.2). Segment B is 2.9 kb in size and codes for VP1 (90 kDa), the RNA-dependent-RNA polymerase (RdRp) (Lombardo *et al.*, 1999) which is covalently integrated to the viral RNA as a genome-linked protein (Kibenge and Dhama, 1997) and is also found as a 90 kDa protein within the virion in its "free" form (Kibenge and Dhama, 1997).

VP4 is a serine protease that uses a Ser^{652}/Lys^{692} catalytic dyad which cleaves Ala-Ala dipeptide bonds (Birghan *et al.*, 2000, Lejal *et al.*, 2000). VP3 is a structural protein which forms Y-shaped trimers on the inner capsid surface of IBDV and has a very basic carboxy-terminal region which is associated with the packaged viral RdRp, VP1 (Hudson *et al.*, 1986, Chevalier *et al.*, 2004). pVP2 is further cleaved to yield mature VP2 (Met^{1}-Ala^{441}, 47 kDa) (Muller and Becht, 1982) and four smaller structural peptides, pep46 (Phe^{442}-Ala^{488}), pep7a (Ala^{489}-Ala^{494}), pep7b (Ala^{495}-

Ala[501]) and pep11 (Ala[502]-Ala[512]) (Da Costa *et al.*, 2002) (Figure 1.2). These peptides remain associated with the capsid (Da Costa *et al.*, 2002) and are involved in cell entry by promoting disruption of host cell membranes (Chevalier *et al.*, 2005, Galloux *et al.*, 2007). Initially, the secondary processing of pVP2 into VP2 was hypothesised to be catalysed by VP4 protease based on the presence of the Ala-Ala bonds (Kibenge *et al.*, 1988). However, Lee *et al.* (2004) demonstrated that the processing of pVP2 was observed in the absence of VP4. It was subsequently shown that the processing at the Ala[441]-Phe[442] bond to release VP2 was carried out by the endopeptidase activity of the capsid protein VP2 involving the Ala[431] residue (Irigoyen *et al.*, 2012).

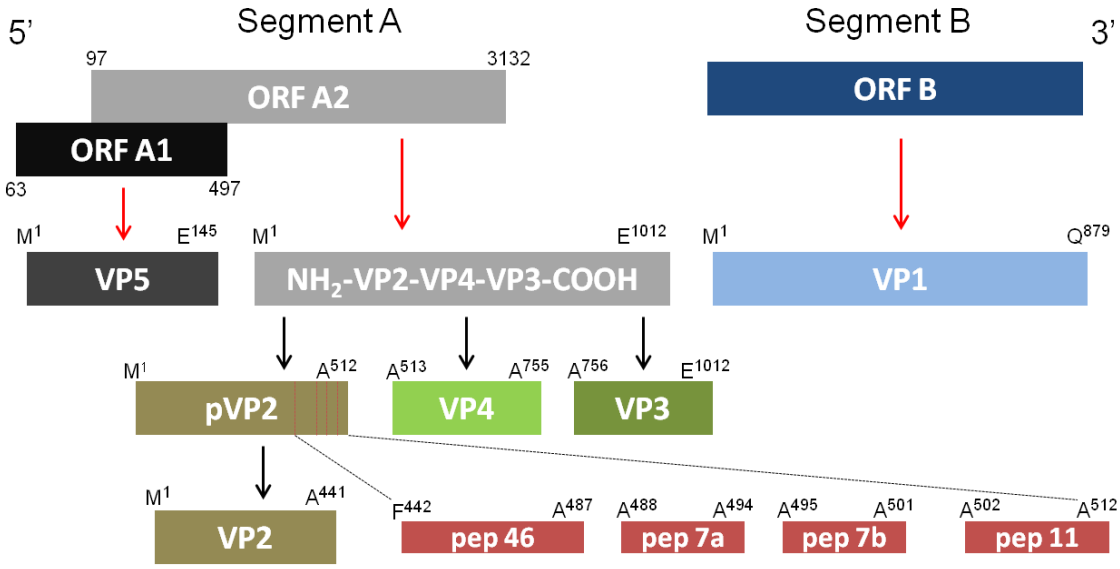

Figure 1.2 Schematic representation of genomic structure and polyprotein processing of IBDV. ORFs, proteins and peptides are depicted as rectangular boxes. Red arrows indicate translation while black arrows indicate cleavage products during polyprotein processing. N-terminal and C-terminal residues are indicated above each protein or peptide and the base pair numbers are indicated above Segment A to portray the overlapping ORFs.

VP2 is a structural protein which forms trimers on the outer capsid of IBDV as protrusions on the surface and is the receptor binding protein (Yip *et al.*, 2007). VP2 is also the primary host-protective immunogen and therefore the primary target for vaccine development (Chen *et al.*, 2005). The crystal structure of IBDV VP2 subviral particle (SVP) (T=1) has been resolved at 2.6 Å (Coulibaly *et al.*, 2005, Garriga *et al.*, 2006, Lee *et al.*, 2006a) and revealed the VP2 subunit folds into a jelly roll fold with three distinct domains; base (B), shell (S) and projection (P) (Figure 1.3A). Domains B and S are well conserved while domain P is highly variable (Coulibaly *et*

al., 2005, Garriga *et al.*, 2006, Lee *et al.*, 2006a) resulting in the continuous emergence of variant IBDV strains (Chen *et al.*, 2005). All three domains take part in trimer formation which resembles an equilateral triangle (Figure 1.3C) with each side measuring 100 Å with a thickness of 25 Å (Coulibaly *et al.*, 2005, Garriga *et al.*, 2006, Lee *et al.*, 2006a). A calcium-binding site is located on the internal surface of the SVP (Figure 1.3B and C) and binds three pairs of symmetry-related Asp^{31} and Asp^{174} in domain S. The Ca^{2+} ion acts as a sealing element for the VP2 trimers (Coulibaly *et al.*, 2005, Garriga *et al.*, 2006, Lee *et al.*, 2006a) by arranging the two Asp residues from three VP2 subunits in an octahedral geometry. In addition, an amphipathic α-helix is found on the C-terminus which mediates interaction between trimers and is important in capsid assembly (Luque *et al.*, 2007) (Figure 1.3B and D).

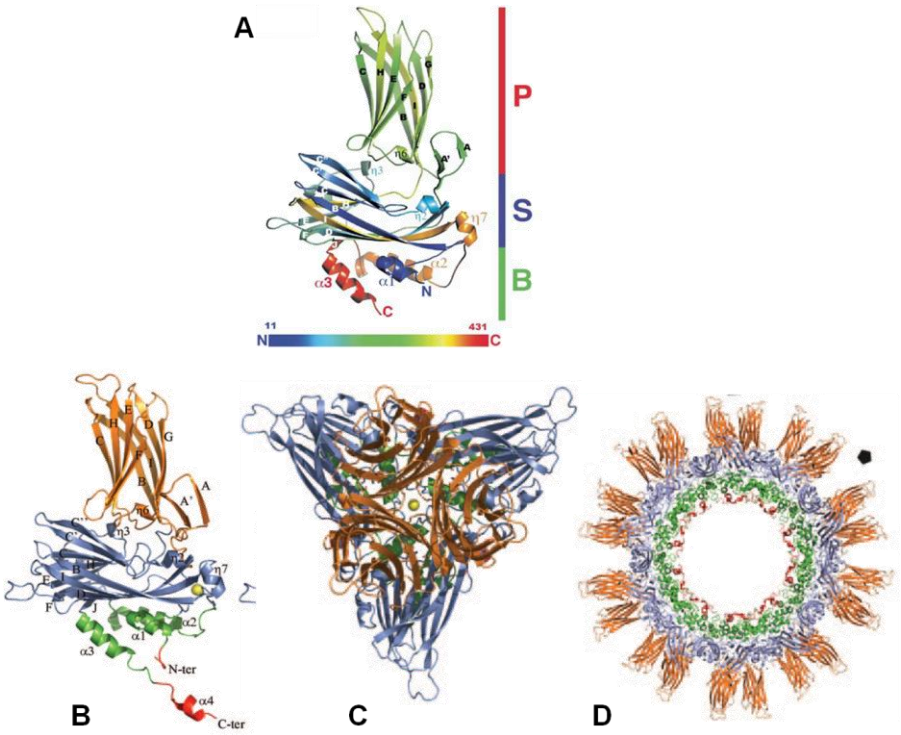

Figure 1.3 The structure of VP2 subunit and VP2 trimer formation. (A) VP2 ribbon structure. The domains are coloured according to the side bar and represent the P domain in red, the S domain in blue and the B domain in green (Coulibaly *et al.*, 2005). **(B)** Ribbon diagram of VP2 showing the amphipathic α-helix on the C-terminus. The three different domains P, S, and B are coloured orange, blue and green respectively. Additional amino acids of the N and C termini are shown in red. The Ca^{2+} ion is represented as a solid sphere in yellow (Garriga *et al.*, 2006). **(C)** VP2 trimer formation. The domains and Ca^{2+} ion are depicted by the same colours as those in panel B (Garriga *et al.*, 2006). **(D)** VP2 SVP ribbon diagram. The domains are represented by the same colours as those in panel B and C and the figure is used to illustrate the position of the amphipathic α-helix (red) which connects the different trimers, (Garriga *et al.*, 2006).

The virus is not easily adapted to cell culture and requires multiple passages (Hassan *et al.*, 1996, van Loon *et al.*, 2002). However, it was demonstrated that altering specific amino acids in the VP2 amino acid sequence allowed for tissue culture adaptation and attenuation of the virus (van Loon *et al.*, 2002). It was therefore observed that VP2 contains tissue culture adaptation determinants. Sequence analysis determined that tissue adaptation resulted in the change of three amino acid residues in the VP2 amino acid sequence (Yamaguchi *et al.*, 1996b, van Loon *et al.*, 2002, Kwon and Kim, 2004). These changes included Glu253His, Asp279Asn and Ala284Thr (Yamaguchi *et al.*, 1996b, Kwon and Kim, 2004). The three amino acid residues are located on the most exposed loops of the P domain which is the site of high variability referred to as the P-loop (Coulibaly *et al.*, 2005). The residues are not involved in the stabilisation of the capsid or in contacts important for the VP2 fold but they engage directly with the cellular receptor on bursal membranes (Coulibaly *et al.*, 2005).

1.2.2 Virulence of IBDV

Two different serotypes of IBDV are distinguished by virus neutralisation tests (McFerran *et al.*, 1980). Serotype I viruses are pathogenic in chickens causing bursal atrophy and immunosuppression while serotype II viruses isolated from turkeys, fowls and ducks are non-pathogenic in chickens. Serotype I viruses are further grouped into four pathotypes based on their virulence, namely classical virulent, antigenic variant IBDV, attenuated and very virulent IBDV (vvIBDV) (Lim *et al.*, 1999).

The classical virulent strains were first discovered in Gumboro in 1960 (Cosgrove, 1962). Clinical signs displayed by chicken infected with this pathotype include inflammation of the bursa and lymphoid necrosis which leads to immunosuppression and 20 to 30% mortality in specific-pathogen-free (SPF) chickens (Lim *et al.*, 1999). In the mid 1980s, vaccinations were seen to fail in different parts of the world. The new isolates emerged due to antigenic drift rendering vaccines made against classical virulent IBDV ineffective to the new isolates (Jackwood and Saif, 1987, Snyder *et al.*, 1992, van den Berg *et al.*, 2000). The new IBDV strain termed antigenic variant IBDV causes rapid and severe bursal atrophy without showing any clinical signs of infection like classical virulent IBDV (Vakharia *et al.*, 1994).

Therefore antigenic variant strains are recognised by their ability to escape cross-neutralisation with antisera against classical virulent IBDV (Lim *et al.*, 1999).

Very virulent IBDV (vvIBDV) strains emerged in the late 1980s and are antigenically similar to classical IBDV (Cao *et al.*, 1998b). However, vvIBDV strains have the ability to break through cellular immunity and is characterised by severe clinical signs and high mortality of up to 60-100% in SFP chickens (Lim *et al.*, 1999). Clinical signs are similar to classical IBDV, but the acute phase is much more severe and widespread in the affected flocks (van den Berg *et al.*, 2000). Attenuated IBDV is produced by adapting classical, variant or vvIBDV pathotypes to cell cultures such as chicken embryo fibroblast (CEF) cells, chicken bursal lymphoid cells and chicken embryo kidney cells through serial passages (Lim *et al.*, 1999). The attenuated IBDV strain is therefore used as a live vaccine due to its reduced virulence (van den Berg *et al.*, 2000).

1.2.3 Pathogenesis and the bursa of Fabricius

The bursa of Fabricius is the target organ for IBDV infection and is also the site for B-cell development in chickens (Kaufer and Weiss, 1980). During embryonic development the bursa starts developing from an epithelial bud in the cloacal region and by embryonic development day 8, B-cell precursors are first observed (Sayegh *et al.*, 1999). B-cell precursors undergo productive immunoglobulin (Ig) gene arrangement and subsequent expression of surface Ig (sIg) (Ratcliffe, 2006). The sIg^- cells undergo apoptosis while the sIg^+ cells continue to divide in the bursal cortex and shortly before hatching migrate to the secondary lymphoid organs and blood across the bursal epithelial basement membrane (Nieminen *et al.*, 2002). In chickens, 3-6 weeks of age, the bursa reaches its maximum development (van den Berg *et al.*, 2000). No new B-cells are produced and therefore chickens are required to survive throughout adult life with these postbursal B-cells (Ratcliffe, 2006).

The general route of IBDV infection is through the oral tract after which it moves into the gut and is transported by phagocytic macrophages to other tissues. Immunofluorescence studies conducted by Muller *et al.* (1979) detected IBDV in the macrophages and lymphoid cells of the cecum 4 h post infection (PI) and in the lymphoid cells of the duodenum and jejunum at 5 h PI. IBDV is also detected in the liver 5 h PI after which IBDV enters the blood stream (Muller *et al.*, 1979). IBDV then

reaches the bursa at 11 h PI and spreads rapidly through bursal follicles causing extensive damage to the medulla and cortical regions as replication of viral particles take place (Tanimura and Sharma, 1997). IBDV enters the bloodstream again and is spread to other lymphoid tissues such as the cecal tonsils and spleen (Sharma *et al.*, 2000).

The acute phase of the disease lasts around 7-10 days during which the bursal follicles are depleted of B-cells and the bursa becomes atrophic (Sharma *et al.*, 2000) resulting in immunosuppression and mortality. Furthermore both cellular and humoral immune responses are compromised (Sharma *et al.*, 2000). Neighbouring virus-free cells undergo apoptosis which is suggested to be the anti-viral mechanism of the host to prevent virus spread (Jungmann *et al.*, 2001). VP2 and VP5 are the only two viral proteins that have been associated with apoptosis induction (Fernandez-Arias *et al.*, 1997, Yao *et al.*, 1998). The resulting effects of the virus vary due to different factors such as the strain of the virus and the age of the infected chicken. Chickens less than 2 weeks old are less susceptible as the bursa is still undergoing development and is therefore protected by maternally derived antibodies. Chickens between the ages of 3-6 weeks old are more susceptible to IBDV because the bursa has reached maximum development while chickens greater than 6 weeks seldom show clinical signs of the disease although they do develop antibodies against the virus (Mahgoub, 2012).

It has been observed that antigenic variant IBDV strains induce bursal atrophy without inflammation while classical virulent strains induce a severe inflammatory response (Sharma *et al.*, 1989) which leads to disease and often death (Muller *et al.*, 1979). The vvIBDV strains induce the same effects as classic or antigenic variant except the rate of replication is greater (van den Berg *et al.*, 2000). T-cells are resistant to IBDV infection (Hirai and Calnek, 1979) and have been shown to reduce replication during the beginning stages of infection (Rautenschlein *et al.*, 2002). The mechanism in which T-cells achieve this is still unknown. The T-cells, however, move into the bursa and reach capacity limit 7 days PI consequently preventing the spread of IBDV and allowing the cells to start recovering (Kim *et al.*, 2000, Sharma *et al.*, 2000).

1.2.4 Control of IBDV infection

IBDV is transmitted by direct contact with contaminated faecal matter, feed and drinking water or by indirect contact with contaminated vectors (van den Berg *et al.*, 2000). The virus is very resistant and persists after thorough cleaning and disinfection (Lukert and Hitchner, 1984). The current control strategy for IBDV is vaccination (Muller *et al.*, 2012). There are currently different vaccines available on the market which include conventional live and inactivated IBDV, genetically live engineered IBDV, subunit vaccines, IBDV immune complex vaccines, DNA vaccines and live viral vector vaccines.

Conventional live and inactivated vaccines are vaccines in which IBDV has been attenuated through serial passages in tissue culture, eggs or embryo-derived tissues and are used to mimic infection in the host target (Schijns *et al.*, 2008, Muller *et al.*, 2012). Because the whole inactive virus is used as the vaccine, no additional adjuvant is required and the vaccine is suitable for mass administration. Unfortunately most commercially available conventional live vaccines are based on classical virulent IBDV (Muller *et al.*, 2012) and therefore other strains such as vvIBDV and antigenic variant IBDV are able to escape neutralisation. The unwanted side-effects of conventional live inactive vaccination include reversion to virulence and moderate to severe bursal atrophy resulting in immunosuppression (Rautenschlein *et al.*, 2005). Nobilis® Gumboro inac (Intervet International B.V., Egypt) and Medivac Gumboro Emulsion (Medion, Indonesia) are examples of inactivated vaccines currently available on the market.

Genetically engineered vaccines are still undergoing development and none have yet reached the market. These vaccines are developed by mutating the IBDV VP2-encoding nucleotide sequence through site-directed mutagenesis (Islam *et al.*, 2001, van Loon *et al.*, 2002, Raue *et al.*, 2004, Noor, 2009, Muller *et al.*, 2012). Reversion to virulence is frequent in this type of vaccine (Raue *et al.*, 2004, Noor, 2009, Muller *et al.*, 2012). Alternately, inter-serotypic vaccines are generated which consist of virulent serotype I and non-pathogenic serotype II strains as the parent viruses (Zierenberg *et al.*, 2004, Muller *et al.*, 2012). This vaccine was established as a possible vaccine candidate as it induced high antibody titres and did not cause damage in the bursa (Muller *et al.*, 2012).

IBDV subunit vaccines are generally based on recombinantly expressed VP2 (Muller *et al.*, 2012) as it contains the major protective neutralising epitopes of IBDV. The subunit vaccine is usually administered with an adjuvant or fused with a protein which enhances immunogenicity (Liu *et al.*, 2005, Muller *et al.*, 2012) such as chicken interleukin-2. Subunit vaccines have been reported to be expressed in *E. coli* (Rong *et al.*, 2007), *P. pastoris* (Fahey *et al.*, 1991) and the baculovirus system (Bayliss *et al.*, 1991). A limiting factor for the subunit vaccine is the way in which it is administered which is intramuscularly. This method of administration can be considered time consuming and expensive especially on large poultry farms where additional booster immunisations are also required.

Immune complex vaccines are a mixture of anti-IBDV specific antibodies obtained from the sera of immunised chickens and live IBD vaccine virus (Whitfill *et al.*, 1995). This type of vaccine is efficient in the presence of maternally derived antibodies (Haddad *et al.*, 1997, Giambrone *et al.*, 2001) and is more effective than live IBDV vaccines (Jeurissen *et al.*, 1998). A comparative study done by Jeurissen *et al.* (1998) showed that an immune complex vaccine delayed virus detection for 5 days and displayed much lower levels of bursal and splenic B-cell depletion in chickens (Jeurissen *et al.*, 1998). A major advantage of this type of vaccine is that it can be administered *in ovo* by commercial egg-injection machines (Muller *et al.*, 2012). A popular commercially available immune complex vaccine is Cevac Transmune IBD containing Winterfield 2512 strain IBDV which specifically targets classical or vvIBDV (Ivan *et al.*, 2005).

DNA vaccines comprise naked DNA which encodes the target gene. The vaccine successfully induced protection against a virulent IBDV infection (Haygreen *et al.*, 2006, Hsieh *et al.*, 2010, Muller *et al.*, 2012). Administration of the vaccine *in ovo* or at 1 day old requires booster immunisation with inactivated vaccine or vectored vaccine as studies report insufficient protective immunity without additional booster immunisations (Haygreen *et al.*, 2006, Park *et al.*, 2009, Muller *et al.*, 2012). A limiting factor is also the method of vaccine administration which is oral through the use of bacteria, namely, *Lactococcus lactis* and *E. coli.* This method of administration has been variably successful possibly due to problems encountered during secretion or translocation of the expressed viral protein across bacterial cell walls (Li *et al.*, 2006, Mahmood *et al.*, 2007, Muller *et al.*, 2012).

Live viral vector vaccines are genetically engineered vaccines which are made up of a donor gene integrated into the genome of a host vector. Immunisation of the genetically modified vector vaccine elicits an immune response against both the donor gene and the vector. VP2 induces protective immunity to IBDV and therefore has been expressed in multiple vector systems such as fowlpoxvirus (Heine and Boyle, 1993), Newcastle disease virus (Huang *et al.*, 2004), herpesvirus of turkey (HVT) (Darteil *et al.*, 1995) and Marek's disease virus (Tsukamoto *et al.*, 1999). Herpesvirus of turkey has been successfully used as a vector vaccine against Marek's disease for many years (Muller *et al.*, 2012) and has therefore been proposed as a vector vaccine for IBD because of its low sensitivity to maternally derived antibodies (Darteil *et al.*, 1995, Tsukamoto *et al.*, 2002, Muller *et al.*, 2012). The Vectormune® HVT IBD + SB-1 vaccine which is currently on the market in some countries specifically targets IBD and Marek's disease. Protection was reported against a challenge with vvIBDV (Le Gros *et al.*, 2009) and antigenic variant IBDV (Perozo *et al.*, 2009). The vectored vaccine demonstrated the best results compared to the other vaccine candidates, mainly due to its ability to escape interference from maternally derived antibodies (Muller *et al.*, 2012). Meeusen *et al.* (2007), however, stated that it may be difficult to maintain the high efficacy of this vaccine due to the continual emergence of very virulent strains of both IBDV and Marek's disease virus.

Therefore it is noted that vaccines which are available are not proficient in controlling all strains of IBDV and all have many shortfalls. It is therefore critical to develop new strategies to help control infection by targeting a different stage in the virus life cycle such as viral entry which is the first step.

1.3 VIRUS ENTRY

A crucial step in the infectious life cycle of a virus is viral entry (Kalia and Jameel, 2011) and the mechanism of entry is mostly based on the structure of the virus (Klasse *et al.*, 1998). Entry of many enveloped viruses is well understood while entry of many non-enveloped viruses still remains unresolved. Enveloped virus entry involves attachment of virus receptor binding proteins to host cell receptors which mediate a succession of events followed by fusion of the viral membrane to the host membrane (Kalia and Jameel, 2011). Fusion of the viral membrane is either directly with the plasma membrane or an internalisation process into endosomes (Kalia and Jameel, 2011). Non-enveloped viruses do not fuse with the endosome membrane

as enveloped viruses do but rather disrupt the membrane to release viral nucleic acids (Kalia and Jameel, 2011). It is therefore important to identify the cellular receptors which viruses use on host cells in order to gain entry.

1.3.1 Enveloped Virus Entry

Enveloped viruses which target animal cells gain entry through direct fusion with the plasma membrane of the host cell (White *et al.*, 1983) or through endocytosis and fusion with the endosomal membrane (Kalia and Jameel, 2011). Viruses which gain entry into host cells through fusion contain viral surface proteins called fusion proteins which are oligomeric integral membrane proteins that can be induced to structurally adopt distinct hydrophilic and hydrophobic states (Hernandez, 1996). They generally have common repeat regions consisting of six amino acid residues which mediate oligomerisation of the proteins and a hydrophobic region, termed the fusion peptide, which can enter the lipid bilayer of the host cell (Gaspar *et al.*, 2001, Da Poian *et al.*, 2005). Fusion is via initial receptor interaction which then triggers a pH dependent or independent conformational change allowing fusion of viral and cellular membranes (Peisajovich and Shai, 2002, Jahn *et al.*, 2003, Kalia and Jameel, 2011). Enveloped viruses of the *Paramyxoviridae* family, which include the measles virus (Table 1.1), gain entry through direct fusion with the plasma membrane with the use of fusion peptides (Figure 1.4).

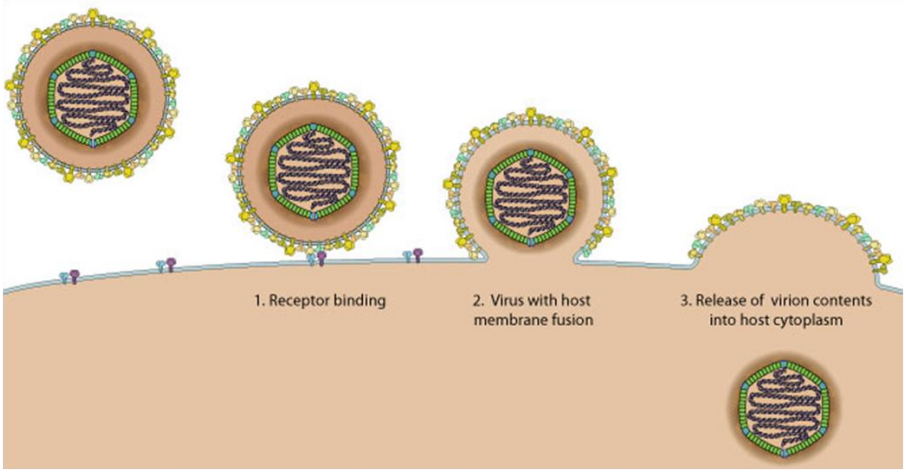

Figure 1.4 Viruses of the Paramyxoviridae family fuse directly with the host plasma membrane to gain entry into host cells. The virus interacts with receptors on host cells **(1)** which triggers a conformational change allowing the viral membrane to fuse with the host cell membrane **(2)** through the use of fusion peptides and releases viral DNA into host cytoplasm **(3)**, (Lee, 2010).

Entry of the Human immunodeficiency virus (HIV) of the *Retroviridae* family (Table 1.1) involves endocytosis and pH independent fusion. Entry is initiated by the interaction of attachment factors with the HIV envelope glycoprotein (Env), which is a highly glycosylated trimer of gp120 and gp41 heterodimers. This interaction brings the envelope in close proximity to the viral receptor, CD4 (Table 1.1) and CCR5 co-receptor which increases the efficiency of infection (Orloff *et al.*, 1991, Wilen *et al.*, 2012). HIV attachment factors include negatively charged cell surface heparan sulfate proteoglycans (Saphire *et al.*, 2001), α4β7 integrin (Arthos *et al.*, 2008, Cicala *et al.*, 2009) and dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN) (Ugolini *et al.*, 1999, Geijtenbeek *et al.*, 2000). Subunit gp120 of Env then interacts with the CD4 binding site of the host cell which causes a rearrangement of three variable loops in gp120. This leads to the formation of a bridging sheet which allows the third phase of HIV entry which is co-receptor engagement (Wilen *et al.*, 2012). Reports have shown that HIV does not fuse directly with the plasma membrane as previously considered, but instead utilises endocytosis before fusion can completely occur (Miyauchi *et al.*, 2009). Reports have shown that HIV-1 infects cells via endocytosis and envelope glycoprotein- and dynamin dependent fusion with endosomes (Miyauchi *et al.*, 2009) (Figure 1.5).

The Influenza virus uses a pH-dependent mechanism of entry and initiates infection by binding to cell surface sialic acid residues (Table 1.1) via interaction with the viral haemagglutinin glycoprotein (Weis *et al.*, 1988). Influenza has been shown to enter host cells via multiple different pathways which include clathrin-dependent and clathrin- and caveolin independent endocytic mechanisms (Nunes-Correia *et al.*, 2004, Rust *et al.*, 2004). Clathrin-mediated endocytosis is initiated as clathrin and associated proteins accumulate on the surface of cellular membranes. The accumulation of these proteins at the surface causes invaginations or pits to form which pinch off into the cell as vesicles through the action of the GTPase dynamin. Vesicles progress to early endosomes and become increasingly acidic as they progress into late endosomes. Caveolin-mediated endocytosis is slower than clathrin-mediated endocytosis and the resulting vesicles do not become acidic. The caveolar pathway is a dynamin and cholesterol dependent pathway (Kalia and Jameel, 2011). Caveolar are micro-invaginations of the plasma membrane which are involved in signal transduction (Anderson, 1998). Internalisation of virus by

endocytosis into caveosomes is initiated by cell stimulation (Ceresa and Schmid, 2000, Sieczkarski and Whittaker, 2002).

Once the virus is internalised into an endosome haemagglutinin, a glycoprotein trimer, experiences an irreversible conformational change at low pH which allows for the insertion of a coiled peptide (Yu *et al.*, 1994) of the haemagglutinin trimer into the membrane (Thorley *et al.*, 2010). This action brings the viral envelope and endosomal membranes in close proximity which assists in membrane fusion (Doms *et al.*, 1985, Chambers *et al.*, 1990, Yu *et al.*, 1994). Viral ribonucleoproteins escape into the cytosol and are imported into the nucleus, where replication occurs (Lakadamyali *et al.*, 2004). Fusion of the viral membrane with the host membrane is therefore the successful route of enveloped virus entry (Figure 1.5). Non-enveloped viruses, however, cannot use this method as they do not possess a membrane. Penetration of the host membranes is therefore used instead.

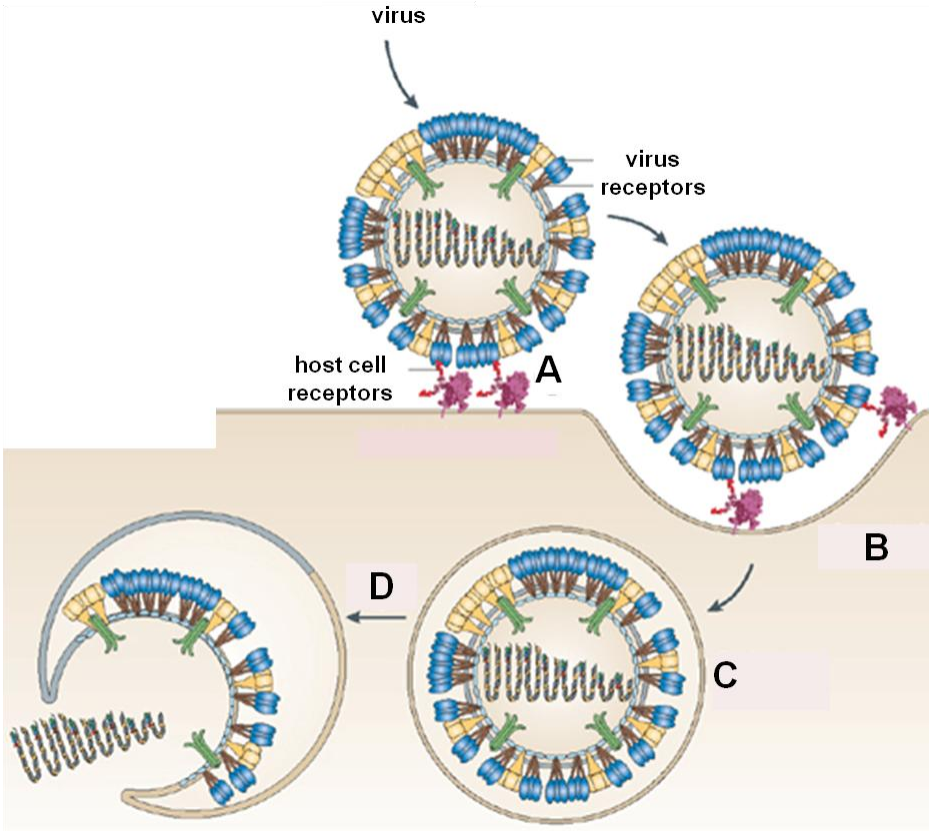

Figure 1.5 Several enveloped viruses use endocytosis and fusion with the endosomal membrane to gain entry into host cells. The virus interacts with the receptor(s), co-receptor(s) and/or attachment factors **(A)** which initiates endocytosis **(B)**. The virus uses a pH dependent or independent mechanism to allow for fusion with the endosomal membrane **(C)** before releasing viral nucleic acids into the cytoplasm **(D)**, adapted from Karlsson Hedestam *et al.* (2008).

1.3.2 Non-enveloped Virus Entry

Non-enveloped viruses use endocytic pathways such as clathrin-coated pits and caveolar pathways to gain access into host cells. These viruses then penetrate membranes of endosomes or caveosomes to release into the host cytoplasm. Conformational change of the virus particles, due to receptor binding and/or low pH conditions, aid in penetration (Hogle, 2002, Rossmann *et al.*, 2002). Four non-enveloped viruses which have been studied extensively and their mechanism of entry understood fairly well include the Poliovirus, Simian virus 40 (SV40), Adenovirus and Bluetongue virus. These viruses use different mechanisms of entry and penetration.

Poliovirus of the *Picornaviridae* family (Table 1.1) causes human poliomyelitis. Cell entry of poliovirus is independent of clathrin and caveolar pathways (Brandenburg *et al.*, 2007), but uses a tyrosine kinase- and actin-dependent, endocytic mechanism instead. Infection is initiated when the virus binds the poliovirus receptor (PVR, or CD155) which results in the virus capsid undergoing a conformational rearrangement (Mendelsohn *et al.*, 1989). The rearrangement causes the exposure of the N-terminus of capsid protein, VP1 and myristoylated auto-cleavage peptide VP4, which are capable of inserting into liposomes (Fricks and Hogle, 1990, Belnap *et al.*, 2000). After insertion these sequences form a transmembrane pore which allows genomic RNA to pass through into the host cytoplasm (Hogle, 2002).

SV40 is a *Polyomavirus* which uses the caveolar pathway (Pelkmans and Helenius, 2002) (Figure 1.6). Endocytosis via the caveolar is followed by a series of complex signalling which results in the release of virus from the caveosome into the cytoplasm of the host cell. The Adenovirus of the *Adenoviridae* family (Table 1.1) is a DNA virus which enters the host cell membrane through endocytosis via clathrin-coated pits (Blumenthal *et al.*, 1986) (Figure 1.7). Low pH conditions in the late endosome cause partial disassembly and a conformational change in the structure of the virus which renders the adenovirus particles hydrophobic and therefore able to permeabilise endosomes and release viral particles (Blumenthal *et al.*, 1986).

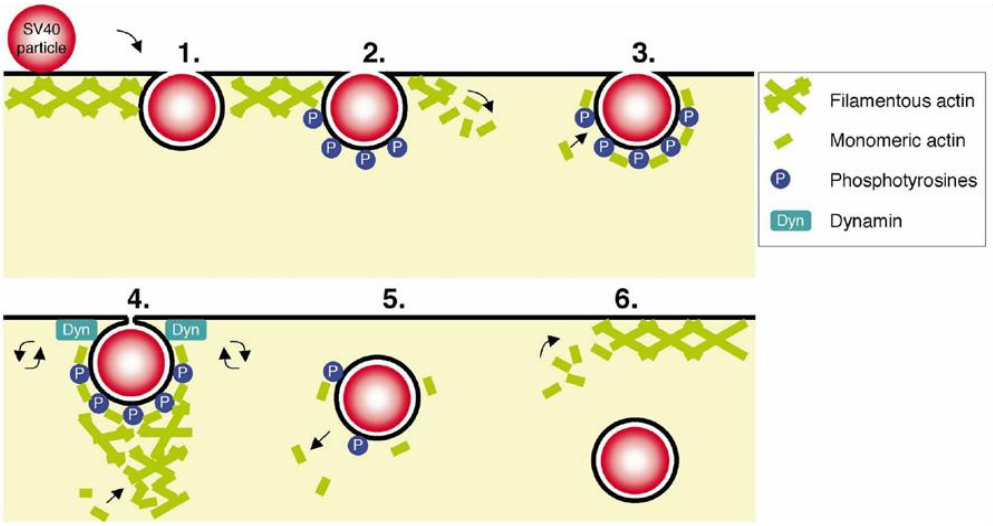

Figure 1.6 Several viruses like SV40 use the caveolar pathway to gain entry into host cells. Caveolae, which are part of the actin cytoskeleton, trap viruses bound to cell surface receptors **(1)** triggering a signal transduction cascade which leads to tyrosine phosphorylation and depolymerisation of the actin cytoskeleton **(2)**. Monomers of actin form an actin patch **(3)** while Dynamin simultaneously moves to the caveolae causing a spontaneous actin polymerisation on the actin patch **(4)**. Caveosomes containing virus are therefore released from the membrane and move into the host cytoplasm **(5)**, allowing the actin cytoskeleton to return back to normal **(6)**, (Pelkmans and Helenius, 2002).

Bluetongue virus of the *Reoviridae* family (Table 1.1) also utilises clathrin-dependent endocytosis for entry (Figure 1.7) and has two capsid proteins, VP2 and VP5. VP5 is structurally similar to class 1 fusion proteins of enveloped viruses and is capable of undergoing pH-dependent conformational changes which allow it to act as a membrane permeabilisation protein that mediates release of viral particles from endosomal compartments into the cytoplasm (Forzan *et al.*, 2004). Table 1.1 shows the receptors used by enveloped and non-enveloped animal viruses.

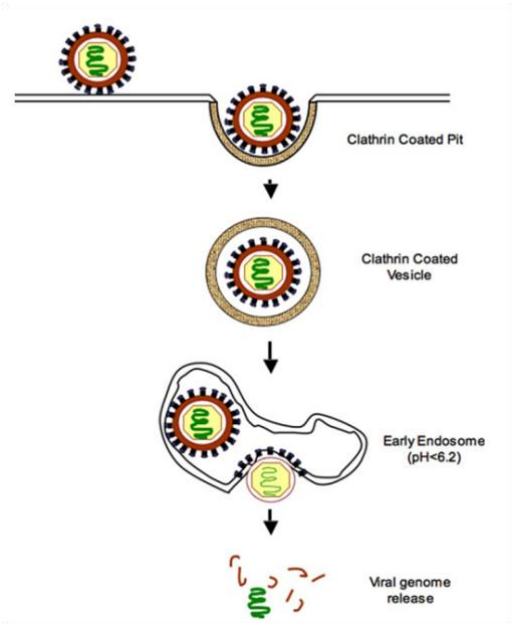

Figure 1.7 Many viruses use clathrin-dependent endocytosis through clathrin-coated pits to gain access into host cells. Clathrin coated pits pinch off into the host cytoplasm through the GTPase dynamin where vesicles progress into early endosomes increasing in acidity. Decrease in pH causes a conformational change of virus which assists fusion or penetration of the endosomal membrane allowing for the release of viral nucleic acids, (Kalia and Jameel, 2011).

1.3.3 IBDV Entry

The mechanism of IBDV entry is still unknown, however, studies have shown that the entry may involve pep46, a structural peptide released from the processing of pVP2 (Galloux *et al.*, 2007). Galloux *et al.* (2007) reported that pep46 is capable of deforming synthetic membranes and inducing pores which can be visualised by electron microscopy. The study also showed that the entry mechanism is dependent on calcium concentration. The lowering of the calcium concentration was found to promote the release of pep46 which induced the formation of pores in the endosomal membrane. The theory therefore is that IBDV gains entry into host cells by two steps which are endocytosis followed by endosome permeabilisation. The low calcium concentration levels within the endosomes causes pep46 to induce pore formation and thus releasing the virus into the host cytoplasm.

IBDV infects mainly the B-cells of the bursa of Fabricius, but has also been shown to infect CEF cells (Yamaguchi *et al.*, 1996a, Rekha *et al.*, 2014), LSCC-BK3 cells, a bursal-derived lymphoblastoid cell line (Ogawa *et al.*, 1998), Vero cells (Kwon and Kim, 2004), macrophages (Khatri and Sharma, 2007), DF-1 cells, an immortalised cell line derived from primary CEF cells (Lin *et al.*, 2007, Rekha *et al.*, 2014) and

DT-40 cells, an avian leukosis virus-induced chicken B cell line (Delgui *et al.*, 2009a). Attenuation of vvIBDV through serial blind passages in tissue culture results in the change of three residues at positions 253, 279 and 284 in VP2 and these residues are believed to engage in receptor binding (Lim *et al.*, 1999, van Loon *et al.*, 2002). This finding suggests that attenuated and very virulent strains use different receptors, however, it was later shown that D78, an attenuated viral strain, blocked the binding of both strains to Vero cells and therefore indicating that both strains utilise the same receptor(s) (Yip *et al.*, 2007).

Initial studies indicated that IBDV targets cells that carry surface IgM (sIgM) (Hirai and Calnek, 1979). However, it was later shown that IBDV could bind sIgM negative cells (Ogawa *et al.*, 1998). Addition of an antibody against sIgM did not inhibit the binding of IBDV to sIgM which further showed that sIgM is not the IBDV receptor (Ogawa *et al.*, 1998). Ogawa *et al.* (1998) treated chicken B-cells with proteases and N-glycosylation inhibitors which reduced IBDV infection suggesting the receptor(s) to contain an N-glycosylated polypeptide(s). Saturation and competitive studies conducted by Nieper and Muller (1996) using CEF cells and chicken B-cells revealed that the two IBDV serotypes have both common and serotype specific receptors (Nieper and Muller, 1996). The study showed both serotypes specifically bound to proteins with molecular masses of 40 kDa and 46 kDa on CEF cells and lymphoid cells in a virus overlay protein binding assay (VOPBA) suggesting common receptor sites of IBDV. A VOPBA using vvIBDV showed virus particles binding to proteins of 70, 80 and 110 kDa which were expressed in LSCC-BK3 cells (Setiyono *et al.*, 2001) and later it was shown that chicken heat shock protein 90 (cHsp90) formed part of the putative cellular receptor which is essential for IBDV entry into DF-1 cells (Lin *et al.*, 2007). Additionally, a study conducted by Delgui *et al.* (2009b) determined that IBDV may also use the α4β1 integrin as a specific binding receptor in avian cells. The research to date on cellular receptor(s) for IBDV has only revealed the size and nature of the receptor and the discovery of cHcp90 was the first study to actually identify a specific molecule.

Virus attachment to host cells is the first step in the virus life cycle and therefore a critical step. Research continues on in attempts to identify the IBDV receptor(s) on host cells which could lead to the development of anti-viral agents which prevent infection of IBDV like the drug compound, PRO 542, successfully developed to control HIV entry into host cells (Jacobson *et al.*, 2000, Shearer *et al.*, 2000).

Table 1.1 Virus pathogenesis and tropism is dependent on the presence of specific receptor(s) or molecules

FAMILY	VIRUS	ENVELOPED /NON-ENVELOPED	RECEPTOR/ CO-RECEPTOR	NUCLEIC ACID	TROPISM	DISEASE
Orthomyxoviridae	Influenza	Env	Sialic acid	dsRNA	Respiratory tract	Influenza
Retroviridae	HIV	Env	CD4, CCR5, CXCR4	dsRNA	Macrophages, T-cells, dendritic cells, microglia brain cells	Lymphadenopathy, AIDS, encephalopathy
Flaviviridae	Hepatitis C	Env	CD81, SR-BI and CLDN1	ssRNA	Hepatocytes	Cirrhosis and hepatocellular carcinomas
	Dengue	Env	Heparan sulfate, HAR and FcγR	ssRNA	Monocytes/ macrophages	Febrile illness, haemorrhagic fever or shock syndrome
Paramyxoviridae	Measles	Env	CD46, second unknown receptor	ssRNA	Respiratory tract mononuclear cells endothelial cells	Acute measles, encephalitis and SSPE (Spillner *et al.*, 2012)
Rhabdoviridae	Rabies	Env	Acetylcholine	ssRNA	Neuronal tissue	Rabies: encephalitis
Picornaviridae	Polio	Non-Env	Polio virus receptor (PVR) or CD155	ssRNA	Epithelial cells neurons	Enteric infection poliomyelitis
	Echovirus 1	Non-Env	Complement inhibitor CD55/DAF	ssRNA	Gastrointestinal tract, other tissues	Febrile illness meningitis
Adenoviridae	Adenovirus	Non-Env	CAR (Ig superfamily) $\alpha v\beta_3$ and $\alpha v\beta_5$ integrins	dsDNA	Epithelial cells, lymphoid cells	Respiratory infection, lymphoid tissues
Reoviridae	Bluetongue	Non-Env	β-adrenergic hormone receptor	dsRNA	Epithelial cells and other tissues	Reproductive/neonatal disease
Polyomaviridae	SV40	Non-Env	MHC Class 1	dsDNA	Mesothelial cells	Tumour and kidney disease
Birnaviridae	IBDV	Non-Env	Unknown	dsRNA	Macrophages, lymphocytes and other tissues	Infectious bursal disease (IBD)

1.4 OBJECTIVES OF THIS STUDY

Infectious bursal disease is an economically important disease of poultry even though there are widely used vaccination programmes in place. Not only does it cause mortality in young chickens, but it also increases the susceptibility of other opportunistic pathogens and a poor vaccination response through immunosuppression (van den Berg *et al.*, 2000, Yuan *et al.*, 2012). Additionally, early vaccination is obstructed by maternally derived antibodies and therefore, new strategies and drugs targeted at different stages of the life cycle are required. The initial and one of the most important steps in the life cycle is receptor binding, which initiates virus entry into host cells. Therefore, identifying the receptor(s) could lead to development of antiviral agents which targets receptor binding. Since VP2 is the receptor binding protein of IBDV, the aim of this study was to identify possible IBDV receptor(s) on the plasma membrane of the bursa of Fabricius using recombinant VP2.

In the present study, a virus overlay protein binding assay (VOPBA) and affinity chromatography was used to determine IBDV receptor(s) with the help of VP2. Therefore the first objective was the production of sufficient VP2 for experimental use. VP2 was therefore heterologously expressed in *E. coli* and *Pichia pastoris*. The VP2 coding sequence which was previously cloned into a T-vector and *E. coli* expression vectors, was subcloned into *P. pastoris* yeast expression vector pPIC9. The recombinantly expressed VP2 protein was purified using three phase partitioning (TPP) and chromatographic techniques. In preparation for the VOPBA and affinity chromatography, the purified VP2 was used to raise polyclonal anti-VP2 antibodies in chickens and used to prepare a VP2-coupled affinity matrix. In addition, VP2 peptides were designed from the VP2 amino acid sequence and used to raise polyclonal anti-VP2 peptide antibodies to compare the specificity of the different antibodies. These results are described in Chapter 2.

The second objective was to identify the VP2-binding proteins and potential IBDV receptor(s). To this end, plasma membrane proteins were isolated from non-infected bursa and used in a modified VOPBA with recombinant VP2 and chicken anti-VP2 antibodies or chicken anti-VP2 peptide antibodies to identify VP2-binding proteins. In addition, all VP2-binding proteins were purified from the plasma membrane preparation on the VP2-coupled affinity matrix and further identified using tandem

mass spectrometry. The results of this part of the study are presented in Chapter 3 while the main findings are discussed in Chapter 4.

CHAPTER 2

RECOMBINANT EXPRESSION AND PURIFICATION OF THE IBDV CAPSID PROTEIN, VP2 AND ANTIBODY PRODUCTION

2.1 INTRODUCTION

A novel strategy to control IBDV infection would be blocking viral entry which is the first step in the virus life cycle (Smith and Helenius, 2004). Virus receptors have been successfully used in the design of antiviral drugs which target virus entry (Altmeyer, 2004) much like anti-HIV T20 (Fuzeon, enfuvirtide) which was the first virus entry inhibitor approved for use as an HIV-1 fusion inhibitor (Walmsley *et al.*, 2003, Hardy and Skolnik, 2004). Several studies using various methods and approaches have been conducted on identifying the IBDV receptor(s). Saturation and competitive studies in chicken embryo fibroblast (CEF) cells and chicken B-cells identified two proteins of 40 and 46 kDa to which IBDV specifically bound (Nieper and Muller, 1996). Saturation experiments involved binding excess radiolabelled IBDV with CEF cells and measuring the affinity of IBDV for receptors on CEF cells. In competition experiments CEF cells were bound to a fixed concentration of radiolabelled IBDV and then incubated with unlabelled IBDV. The virus ability to competitively bind CEF cells was then determined. Later, Ogawa *et al.* (1998) demonstrated the ability of IBDV to infect LSCC-BK3 cells (chicken lymphoblastoid cell line) and observed that treatment of the cells with proteases and N-glycosylation inhibitors prevented infection, suggesting that the receptor(s) contain(s) an N-glycosylated protein necessary for infection. A virus overlay protein binding assay (VOPBA) showed that IBDV specifically bound to proteins with molecular masses of 70, 80 and 110 kDa on LSCC-BK3 cells (Setiyono *et al.*, 2001). Whole virus was used in these studies to determine which proteins IBDV bound to on IBDV susceptible cells.

Previous studies have only identified the size or nature of the host cellular receptor(s) and it was only later that chicken heat shock protein 90 (cHsp90) was identified as a possible cellular receptor or forming part of the cellular receptor (Lin *et al.*, 2007). This study made use of DF-1 cells from a spontaneously immortalised cell line, in turn derived from primary CEF cells. Recombinantly expressed IBDV subviral particles (SVPs), instead of whole virus, and monoclonal anti-SVP

antibodies were used in a VOPBA and in affinity chromatography to identify possible receptor(s). The SVPs are formed by VP2 assembly and show the same immunogenicity as IBDV particles (Coulibaly *et al.*, 2005, Lin *et al.*, 2007). Even though several studies have been conducted to identify the IBDV receptor(s) on bursal cells, little is known about the nature of the receptor(s) or the possible existence of co-receptors. The proteins of different cell lines which bind to IBDV have been reviewed by Zhu *et al.* (2008) and are summarised in Table 2.1.

Table 2.1 Summary of IBDV binding proteins of different cell lines to date

CELL LINE	PROTEIN IDENTITY/ MOLECULAR MASS (kDa)	STUDY CONDUCTED BY
CEF B-lymphocytes	40 and 46	Nieper and Muller (1996)
LSCC-BK3	70, 80 and 110	Setiyono *et al.* (2001)
DF-1	cHsp90	Lin *et al.* (2007)

Since SVPs were used to identify cHsp90 as forming part of the cellular receptor, VP2 is a good substitute for the identification of IBDV receptor(s) on bursal cell membranes. Therefore the aim of this part of the study was to recombinantly express VP2 and produce chicken anti-VP2 antibodies for use in a VOPBA and by VP2- affinity chromatography as will be described in Chapter 3. To this end the VP2 coding sequence was sub-cloned and heterologously expressed in bacterial and eukaryotic expression systems and antibodies produced against VP2 and a VP2 peptide. The VP2 coding sequence amplified from IBDV segment A cDNA was previously cloned into a T-vector and sub-cloned into pGEX-4T-1 and pET-32a expression vectors. In the present study bacterial expression of VP2 was achieved using the pET-32a construct by auto-induced expression in *E. coli* and the recombinant VP2 was affinity purified via the His-tag and used to raise polyclonal antibodies in chickens. The VP2 coding sequence was additionally sub-cloned into the pPIC9 yeast expression vector and expression carried out in *P. pastoris* to obtain a higher yield of purified VP2. VP2 expressed in *P. pastoris* was purified using three phase partitioning (TPP) and molecular exclusion chromatography (MEC) and was coupled to an affinity matrix for the purification of chicken anti-VP2 antibodies.

2.2 MATERIALS

General molecular biology: The following reagents were obtained from Fermentas (Vilnius, Lithuania): NotI[a], EcoRI, SacI O'GeneRuler™ 1 kb DNA ladder, MassRuler™ DNA ladder mix, *Taq* DNA polymerase, T4 DNA ligase, 10 mM dNTP mix, shrimp alkaline phosphatase, TransformAid™ bacterial transformation kit, GeneJET™ plasmid miniprep kit and PageRuler™ prestained protein ladder. The peqGOLD gel extraction kit was purchased from PEQLAB Biotechnologie (Erlangen, Germany) and the DNA clean and concentrator kit™ from Zymo Research (Orange, USA). Tetracycline, ampicillin and isopropyl thioglucopyranoside (IPTG) were purchased from Sigma-Aldrich-Fluka (Steinheim, Germany) and urea from Merck Biosciences (Damstadt, Germany). Expression vector, pPIC9, was provided as a glycerol stock by Dr P. Vukea [University of KwaZulu-Natal, (UKZN)]. In previous studies in the laboratory, the mature VP2 coding region was cloned into pET-32a and pGEX-4T-1 expression vectors and transformed into *E. coli* BL21(DE3) cells and in *E. coli* BL21 cells respectively. Both were available as glycerol stocks.

General biochemistry: BioTrace™ NT nitrocellulose membrane was purchased from PALL Corporation (New York, USA) and BCA™ protein assay kit from Pierce (Rockford, USA). Poly-Prep® chromatography columns and Oriole™ fluorescent gel stain were purchased from Bio-Rad (Hercules, USA) and Sephacryl™ S300 HR, SP-Sephadex C-25 and rabbit anti-IgY-horse radish peroxidase (HRPO) from Sigma-Aldrich-Fluka (Steinheim, Germany). Filter paper was obtained from Whatman (Middlesex, UK) and Amicon Centricon® centrifugal concentrators were purchased from Millipore (Billerica, USA). Nunc Maxi Sorp™ 96-well microtiter plates were from Nunc products (Roskilde, Denmark). Ni-NTA His-bind resin and mouse anti-His tag monoclonal antibody were purchased from Novagen (Damstadt, Germany). Horse anti-mouse IgG-HRPO conjugate was purchased from Cell Signaling Technology® (Boston, USA), polyethylene glycol (PEG) 6000 from Merck Biosciences (Damstadt, Germany) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), dithiothreitol (DTT) and bovine serum albumin (BSA) were purchased from Roche (Mannheim, Germany). Chickens used for immunisations were obtained from UKZN Ukulinga research and training farm and approval for antibody production obtained from the UKZN Animal Research Ethics Committee

[a] Nomenclature according to Roberts *et al.* (2003) where gene names are in italics and restriction enzymes are not.

(reference number 36/11/Animal). All other general reagents and chemicals were obtained from Sigma-Aldrich-Fluka (Steinheim, Germany) and Merck Biosciences (Damstadt, Germany).

Antibody preparation: The peptides designed for antibody production were synthesised by GL Biochem (Shanghai, China). Freund's complete and incomplete adjuvants, Sephadex® G-10 and Sephadex® G-25 resins, maleimidobenzoyl-A/-hydroxysuccinimide ester (MBS), rabbit albumin and Ellman's reagent were purchased from Sigma-Aldrich-Fluka (Steinheim, Germany). Aminolink® and Sulfolink® coupling resins were purchased from Pierce Perbio Science (Erembodegem, Belgium).

2.3 METHODS

2.3.1 Expression of VP2 in *E. coli*

2.3.1.1VP2 expression using pGEX-4T-1

The VP2 coding sequence was previously cloned into the pGEX-4T-1 vector (Figure 2.1) between the EcoRI and NotI sites. Glycerol stocks of *E. coli* BL21 cells containing recombinant pGEX-4T-1 were three-way streaked on 2xYT agar plates [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 1.5% (w/v) agar, 86 mM NaCl] containing 50 µg/mL ampicillin and grown inverted overnight at 37°C. A single colony was thereafter inoculated in 2xYT broth containing 50 µg/mL ampicillin (2xYT-Amp) (10 mL) and the cultures were grown overnight with shaking at 37°C. The overnight culture was diluted to 100 mL using fresh 2xYT-Amp and grown at 37°C with shaking until an OD_{600} between 0.4 and 0.6 was reached. Cultures were induced for expression by the addition of IPTG to a final concentration of 1 mM and incubation continued at 37°C with shaking for another 4 h. Ampicillin (50 µg/mL) was added to the culture every hour during expression to maintain selective pressure for transformed *E. coli*. After 4 h the cells were pelleted by centrifugation (5 000 x *g*, 5 min, RT) and resuspended in lysis buffer [PBS (100 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), 0.1% (v/v) Triton X-100, 1 mg/mL lysozyme] for 30 min at 37°C and subsequently frozen at -20°C.

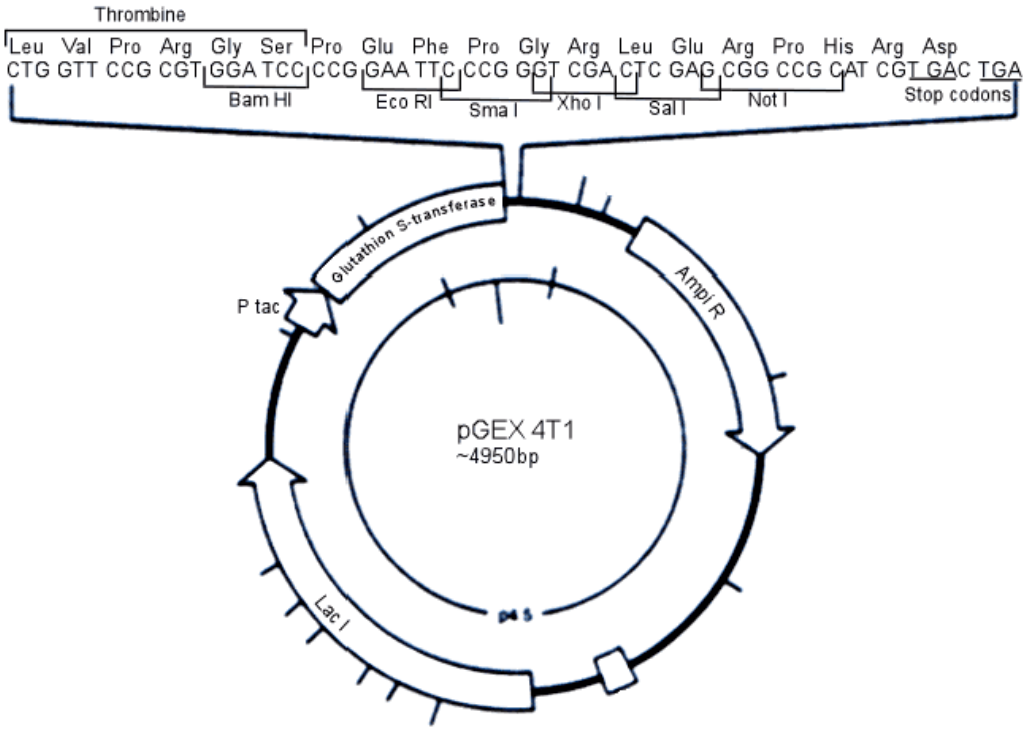

Figure 2.1 Structure of pGEX-4T-1 expression vector. The vector is 4 950 bp in size and contains an ampicillin resistance marker (*Ampi R*), lac promoter (*Lac I*), tac promoter (*P tac*), a *glutathione S-transferase* gene and a multiple cloning site (MCS) in which the VP2 coding sequence was cloned between the EcoRI and NotI restriction sites. The MCS also has a thrombin cleavage site which allows for the GST-tag to be cleaved from the recombinant protein after expression.

2.3.1.2VP2 expression using pET-32a

The VP2 coding sequence was previously cloned into the pET-32a vector (Figure 2.2) between the EcoRI and NotI sites. Glycerol stocks of *E. coli* BL21(DE3) cells containing recombinant pET-32a were three-way streaked on 2xYT agar plates containing 50 µg/mL ampicillin and grown inverted overnight at 37°C. Expression was carried out by either auto-induction or IPTG induction. Auto-induced expression was carried out at 30°C or 37°C overnight or at 16°C or 25°C over 24 h. A single recombinant colony was inoculated in Terrific broth [1.2% ((w/v)) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4] containing 50 µg/mL ampicillin with shaking. Cultures were centrifuged (5 000 x *g*, 5 min, RT), the cell pellet resuspended in 1/10 culture volume of lysis buffer and frozen at -20°C.

Expression induced by IPTG was carried out by inoculating 10 mL of 2xYT-Amp with a single recombinant colony and allowing the cultures to grow overnight with shaking at 37°C. The overnight culture was diluted to 100 mL with fresh 2xYT-Amp broth

and grown at 37°C with shaking until an OD_{600} between 0.4 and 0.6 was reached. Expression was induced by the addition of IPTG to a final concentration of 1 mM and the cultures were further incubated for 4 h. Non-induced cultures were also incubated for 4 h as negative controls. The cells were harvested by centrifugation (5 000 x *g,* 10 min, RT) and the cell pellet was resuspended in 1/10 culture volume of lysis buffer at 37°C for 30 min and frozen at -20°C. All lysates were thawed and disrupted by sonication before analysis by reducing SDS-PAGE and stained with Coomassie blue R-250 (Section 2.3.5.2) followed by western blotting (Section 2.3.5.3).

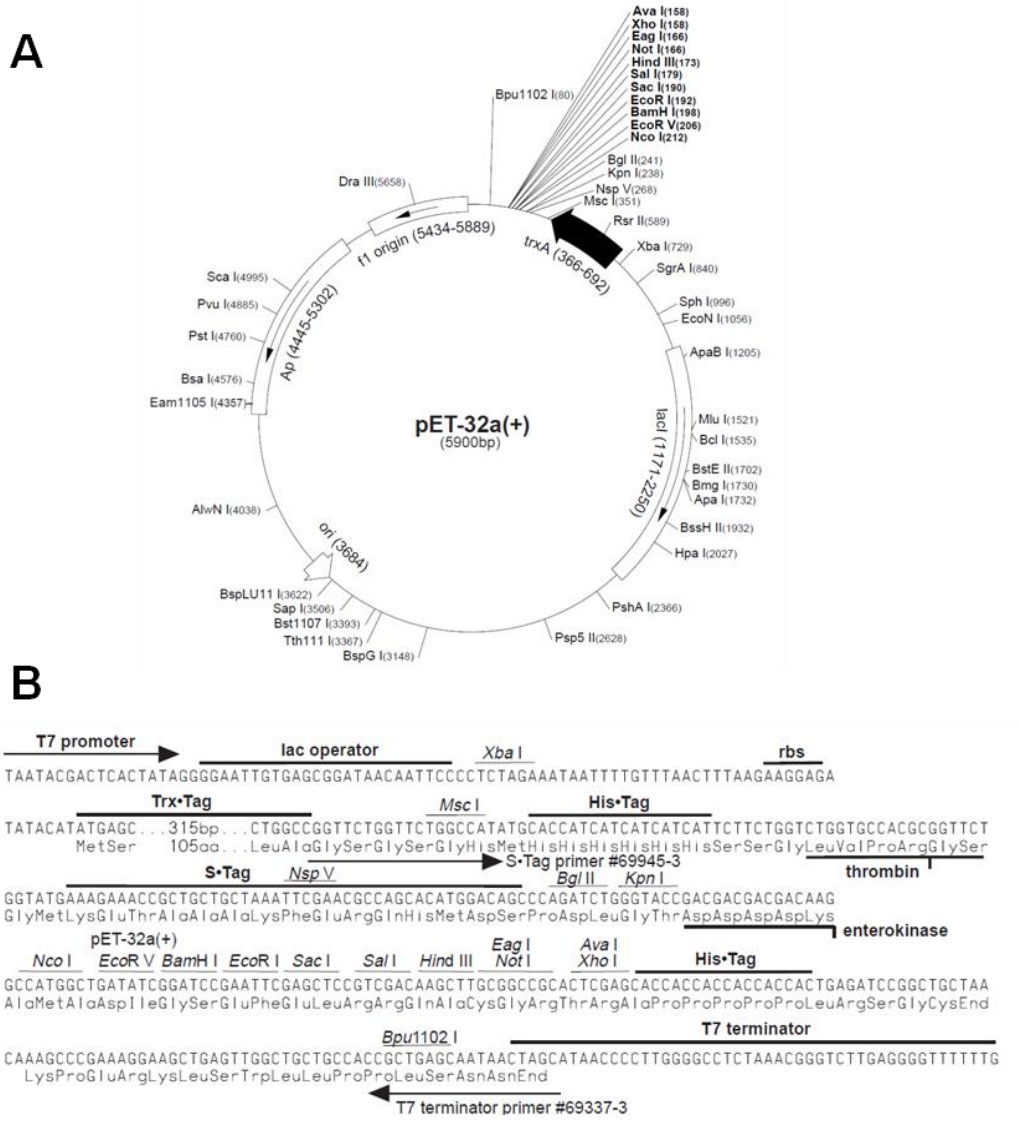

Figure 2.2 Structure of the pET-32a expression vector and sequence of the pET-32a MCS. (A) Schematic map of pET-32a vector. **(B)** Nucleotide sequence of the MCS of pET 32a. The vector contains Trx-Tag, His-Tag and S-tag coding sequences, a MCS in which the VP2 coding sequence was cloned between the NotI and EcoRI restriction sites, an f1 origin of replication, ampicillin resistance gene (*Ap*), lacI promoter (*lacI*) and a T7 promoter and transcription start codon.

2.3.1.3Solubilisation of inclusion bodies

Recombinant VP2 expressed in *E. coli* BL21(DE3) cells was analysed for solubility. The lysates containing expressed proteins were separated into soluble and insoluble protein fractions through high speed centrifugation (14 000 x *g*, 30 min, 4°C). The pellet (inclusion bodies) and supernatant (soluble fraction) were analysed by SDS-PAGE with Coomassie blue R-250 staining (Section 2.3.5.2). Inclusion bodies were solubilised according to Sijwali *et al.* (2001) with a few modifications. Inclusion bodies containing VP2 were washed three times in wash buffer (2 M urea, 0.02 M Tris-HCl, 1% (v/v) Triton X-100, pH 8.0) with centrifugation between washes (14 000 x *g*, 30 min, 4°C). The final pellet was resuspended in solubilisation buffer (8 M urea, 0.02 M Tris-HCl, 0.5 M NaCl, pH 8.0) and incubated at 4°C overnight. Undissolved debris was removed by centrifugation (14 000 x *g*, 30 min, 4°C) before the solubilised proteins were analysed by SDS-PAGE stained with Coomassie blue R-250 (Section 2.3.5.2).

2.3.2 Purification of recombinant VP2 from *E. coli* expression systems

2.3.2.1Purification of VP2 under denaturing conditions using a nickel column

The His-tagged VP2 expressed as insoluble proteins using the pET-32a system. Therefore solubilised inclusion bodies were purified under denaturing conditions on a nickel column. The Ni-NTA His-Bind resin (Novagen, Germany) (2 mL) was packed into a 10 mL Poly-Prep® chromatography column (Bio-Rad, USA) and prepared according to the manufacturer's instructions. The column was equilibrated with 10 column volumes of equilibration buffer (8 M urea, 0.02 M Tris, 0.5 M NaCl, 0.02 M imidazole, 0.001 M β-mercaptoethanol, pH 8.0) under gravitational flow-rate. The solubilised proteins were incubated overnight at 4°C with the resin by end-over-end rotation, after which the unbound proteins were collected from the column. The column was washed with 10 column volumes of wash buffer (8 M urea, 0.02 M Tris, 0.5 M NaCl, pH 8.0) and bound proteins were eluted from the column with elution buffer (8 M urea, 0.02 M Tris, 0.25 M imidazole, pH 8.0) in 0.5 mL fractions. Eluted fractions were analysed by SDS-PAGE stained with Coomassie blue R-250 (Section 2.3.5.2) and analysed by western blotting (Section 2.3.5.3). All eluted fractions containing VP2 were pooled and all wash fractions containing VP2 were pooled separately and further purified using ion exchange chromatography (IEC).

2.3.2.2Purification of VP2 from wash fractions using IEC

Wash fractions containing VP2 from the affinity purification of recombinantly expressed His-tagged proteins were pooled and further purified using cation-exchange chromatography on a SP-Sephadex C-25 cation-exchange column (Himmelhoch, 1971). The pooled wash fractions were prepared for IEC by two dialysis steps in IEC buffer (0.1 M Na-citrate, pH 5.9) with stirring at 4°C for 48 h and thereafter concentrated against PEG M_r 20 000. The column (25 x 150 mm, 30 mL/h, RT) was packed and prepared according to the manufacturer's instructions and equilibrated with 10 column volumes of IEC buffer. The concentrated sample was loaded onto the column followed by washing with IEC buffer. The A_{280} readings of the wash fractions (1 mL) were monitored to determine when all unbound proteins were completely eluted. Once the A_{280} reached baseline, bound proteins were eluted using a step-wise gradient of NaCl (50, 100 and 500 mM NaCl in IEC buffer). One mL fractions were collected and monitored for protein by measuring A_{280}. Elution peaks were analysed by SDS-PAGE followed by staining with silver nitrate (Section 2.3.5.2).

2.3.2.3On-column refolding and affinity purification of VP2

Solubilised His-tagged VP2 was subjected to on-column refolding on a nickel column. The refolding of solubilised VP2 was performed by using a linear urea gradient from 8.0 M urea to 0 M urea. The column was washed starting with the washing buffer (8 M urea, 0.02 M Tris-HCl, 0.5 M NaCl, pH 8.0) and finishing with refolding buffer (0.02 Tris-HCl, 0.5 M NaCl, pH 8.0) with a total gradient volume of 40 mL at a flow rate of 0.5 mL/min. The refolded VP2 was eluted using elution buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.25 M imidazole, pH 8.0) and collected in 0.5 mL fractions before analysis by SDS-PAGE, stained with silver nitrate (Section 2.3.5.2).

2.3.2.4Electro-elution of VP2 and high molecular mass proteins

Electro-elution was used to purify high molecular mass proteins observed expressing in the *E. coli* system, for further identification. Electro-elution was carried out according to Acil *et al.* (1997) with minor modifications. Lysates containing expressed proteins were separated on 10% reducing SDS-PAGE (Section 2.3.5.2) and protein bands were visualised by staining with 0.3 M $CuCl_2$ for 10 min. Protein bands of interest were excised and destained by repeated washes (3 x 10 min) in

destain buffer (0.25 M Tris-HCl, 0.25 M EDTA, pH 8.0) with gentle shaking. Gel slices were equilibrated in electrophoresis buffer (20 mM Tris-HCl, 1.7 mM SDS, pH 8.5) for 5 min, cut into small pieces and placed into an electro-elution chamber and eluted at 80 V for 16 h at RT using the Electro-Eluter Concentrator (CBS Scientific, California, USA). The eluted proteins were analysed by SDS-PAGE, stained with silver nitrate (Section 2.3.5.2).

2.3.3 Expression of VP2 in *P. pastoris*

2.3.3.1Sub-cloning of the VP2 coding region into pPIC9 yeast expression vector

The *E. coli* JM109 glycerol stocks containing recombinant pGEX-4T-1-VP2 and pPIC9 vector (Figure 2.3) were separately three-way streaked on 2xYT agar plates containing 50 µg/mL ampicillin and grown inverted overnight at 37°C. A single colony was used to inoculate 10 mL 2xYT-Amp and grown overnight at 37°C with shaking. The recombinant pGEX-4T-1-VP2 and non-recombinant pPIC9 plasmids were isolated from the overnight cultures using the GeneJET™ plasmid miniprep kit (Fermentas, Lithuania) according to the manufacturer's instructions with minor modifications. Instead of eluting with 50 µL, the elution step was done twice with 25 µL. Purified plasmid DNA was analysed on a 1% *(*(w/v)*)* agarose gel (Section 2.3.5.1) to determine purity.

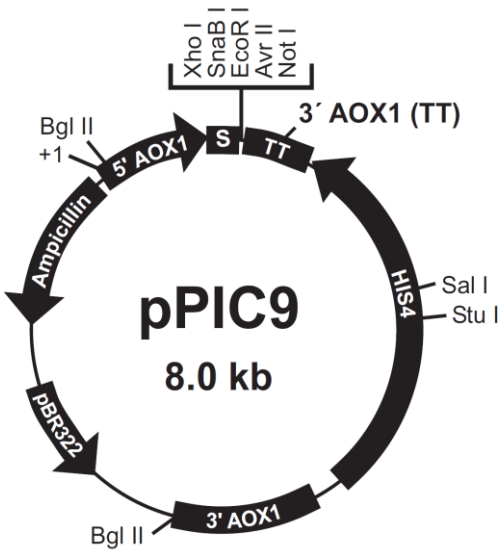

Figure 2.3 Structure of pPIC9 yeast expression vector. The vector contains a ColE1 origin of replication (pBR322), 3' AOX fragment (3' AOX1), a HIS4 gene, which is used for selection by complementation in the his4 yeast strain, c-myc epitope (TT), MCS in which the VP2 coding sequence was cloned between the EcoRI and NotI restriction sites, α-factor secretion signal (S), 5' AOX fragment (5' AOX1) and an ampicillin resistance gene. The vector size is 8.0 kb.

The recombinant pGEX-4T-1-VP2 and non-recombinant pPIC9 plasmid DNA were subjected to a double restriction digest with EcoRI and NotI to release the VP2 insert cDNA and prepare the pPIC9 vector for ligation, respectively. The double restriction was carried out in a 30 µL reaction containing 10 x buffer O (3 µL), plasmid DNA (25 µL), EcoRI (1 µL or 10 U) and NotI (1 µL or 10 U). The digestion reaction was incubated overnight at RT and analysed on a 1% (w/v) agarose gel (Section 2.3.5.1) to assess digestion. The enzymes in the reaction tube for the digestion of pPIC9 vector were deactivated by incubation at 65°C for 15 min prior to dephosphorylation. The dephosphorylation reaction, containing 10 x reaction buffer (3 µL), digestion reaction mix (26 µL) and shrimp alkaline phosphatase (1 µL) was incubated for 1 h at 37°C. The shrimp alkaline phosphatase was deactivated by incubation at 65°C for 15 min and the dephosphorylated vector was purified using the DNA clean & concentrator™ kit (Zymo Research, USA) according to the manufacturer's instructions.

The remaining reaction mix of the digested recombinant pGEX-4T-1 plasmid DNA was electrophoresed on a 1% (w/v) agarose gel and the band corresponding to the VP2 insert cDNA was extracted from the gel using the gel extraction kit (PEQLAB Biotechnologie, Germany) according to the manufacturer's instructions. Purified VP2 cDNA was ligated into pPIC9 vector using T4 DNA ligase (Fermentas, Lithuania) according to the manufacturers' instructions. Negative controls omitting either insert

DNA or T4 DNA ligase were also included. The ligation mixture was incubated overnight at 4°C and the ligation mixtures were used to transform *E. coli* JM109 cells using the TransformAid™ bacterial transformation kit (Fermentas, Lithuania) according to the manufacturer's instructions. The transformed cells were grown overnight at 37°C on 2xYT agar plates containing 50 µg/mL ampicillin. Colonies were screened for recombinant plasmids by colony PCR using AOX vector primers (forward 5'-GACTGGTTCCAATTGACAAG-3' and reverse 3'-GCAAATGGCATTCTGACATCC-5') in a PCR reaction mix containing 1 x PCR buffer, 2.5 mM $MgCl_2$, 0.25 µM AOX forward primer, 0.25 µM AOX reverse primer, 0.5 mM dNTP mix and 0.25 U *Taq* polymerase. Amplification was carried out as follows: 94°C (5 min) for initial denaturation, then 30 cycles of 94°C (30 s), 55°C (30 s) and 72°C (1 min) for denaturation, annealing and extension followed by final extention at 72°C (7 min).

A single recombinant colony was used to inoculate 2xYT broth containing 50 µg/mL ampicillin and grown overnight with shaking at 37°C. The pPIC9-VP2 plasmid DNA was purified using the GeneJET™ plasmid miniprep kit (Fermentas, Lithuania) and analysed on a 1% (w/v) agarose gel (Section 2.3.5.1) before linearisation of the recombinant plasmid DNA with SacI to allow for integration into the *P. pastoris* genome. Transformation of the pPIC9 recombinants were carried out according to Wu and Letchworth (2004). Briefly, *P. pastoris* GS115 cells were grown in 500 mL yeast peptone dextrose medium (YPD) [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] at 30°C overnight, with shaking until an OD_{600} of between 1 and 2 was reached. The GS115 cells were pelleted by centrifugation (708 x *g*, 20 min, 4°C) and resuspended in 400 mL resuspension buffer (100 mM lithium acetate, 10 mM DTT, 0.6 M sorbitol, 10 mM Tris-HCl buffer, pH 7.5) and incubated for 30 min at RT. The cells were again pelleted (708 x *g*, 20 min, 4°C) and washed with ice-cold 1 M sorbitol (45 mL) three times with centrifugation between washes (708 x *g*, 20 min, 4°C) before resuspending the final pellet in ice-cold 1 M sorbitol (1.5 mL).

The prepared *P. pastoris* GS115 cells (200 µL) were added to linearised pPIC9-VP2 (2 µL) and allowed to incubate on ice for 5 min. The mixture was transferred to pre-chilled 2 mm electroporation cuvettes (BioRad, USA) and electroporated (1.5 kV, 25 µF, 186 Ω) using a BioRad Gene Pulser™ electroporator (BioRad, USA). Immediately after electroporation, 1 mL ice cold 1 M sorbitol was added to the cells and 200 µL were plated onto minimal dextrose (MD) plates [1.34% (w/v) YNB, 0.00004% (w/v) biotin, 2% (w/v) dextrose (glucose), 15 g/L bacteriological agar] containing 50 µg/mL ampicillin and

grown for four days at 30°C. A colony PCR was performed using AOX vector primers to screen for recombinants (Ayra-Pardo *et al.*, 1998). Positive clones were inoculated in YPD and grown overnight with shaking at 30°C and stored in sterile 80% (v/v) glycerol at -80°C until required for expression.

2.3.3.2 Yeast expression of VP2 using pPIC-9

Glycerol stocks of *P. pastoris* GS115 cells containing the pPIC9 vector were three-way streaked on YPD plates containing 50 µg/mL ampicillin. The plates were incubated for 48 h at 30°C and single colonies were used to inoculate YPD medium (50 mL) and grown for 48 h at 30°C with shaking to saturation. Buffered medium glycerol yeast (BMGY) [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.5, 1.34% (w/v) yeast nitrogen base without amino acids] (450 mL) was inoculated with the YPD culture (50 mL) and grown for 48 h at 30°C with shaking until an OD_{600} of 3 to 6 was reached. The cells were pelleted by centrifugation (708 x *g*, 10 min, 4°C) and the supernatant discarded. The pellet was checked to ensure an off-white homogenous pellet which indicates lack of contamination.

The pellet was resuspended in 500 mL buffered minimal medium (BMM) [100 mM potassium phosphate buffer, pH 6.5, 1.34% (w/v) yeast nitrogen base without amino acids, 0.0004% (w/v) biotin, 5% (v/v) methanol], transferred to sterile baffled flasks and covered with three layers of sterile cheesecloth to facilitate aeration during expression. Expression was continued for 10 days with shaking at 30°C with the daily addition of 0.5% (v/v) methanol for the duration of expression. Cells were pelleted by centrifugation (5 000 x *g*, 10 min, 4°C) and the supernatant retained. The pellet was used in another round of expression by resuspending in BMM (500 mL) as long as the pellet remained uncontaminated. The supernatant was analysed by SDS-PAGE, stained with silver nitrate (Section 2.3.5.2) and stored at -20°C.

2.3.3.3 Purification of VP2 by three phase partitioning (TPP) and molecular exclusion chromatography (MEC)

Three phase partitioning (Pike and Dennison, 1989) was the initial method used for purification and concentration of VP2 expressed in yeast. Briefly, the supernatants (500 mL) were filtered (Whatman No. 4 filter paper) and tertiary-butanol added to a

final concentration of 30% (v/v). Ammonium sulfate [40% (w/v)] was added and stirred until completely dissolved. This mixture was centrifuged (6000 x *g*, 10 min, 4°C) using a spin-out rotor (JS-7.5) in a Beckman Coulter centrifuge to facilitate separation into three distinct layers: a lower aqueous layer, an upper tertiary butanol layer and a precipitated protein layer at the interface. The precipitated protein layer was collected and dissolved in a minimal volume of PBS and dialysed against two changes of PBS for 48 h at 4°C to remove t-butanol. The dialysed sample was concentrated using PEG 20 000 until the final volume was approximately 5-10 mL before analysis by SDS-PAGE, stained with silver nitrate (Section 2.3.5.2). The concentrated sample was further purified by MEC.

For MEC a Sephacryl S300 HR column (25 x 840 mm, RT) was calibrated at a flow rate of 25 mL/h using 6 mg/mL blue dextran (2000 kDa) and 15 mg/mL each of BSA (68 kDa), ovalbumin (45 kDa) and myglobin (16.7 kDa) dissolved in 5 mL MEC buffer (0.15 M NaH_2PO_4, 0.05 M NaCl, pH 7.0) and a calibration curve generated by monitoring and recording the A_{280} readings. The availability constant (K_{av}) for each protein was determined, where the elution volume (V_e) of blue dextran represents the void volume (V_o) and V_t is the total column volume. A Fischer's plot relating K_{av} to log M_r is presented in Figure 2.4. The column was equilibrated with one column volume of MEC buffer before applying the TPP protein sample (approximately 5 mL) to the column. Eluted fractions were monitored by measuring the A_{280} and thereafter analysed by SDS-PAGE, stained with silver nitrate (Section 2.3.5.2). Fractions containing VP2 were pooled and concentrated using Centricon® centrifugal concentrators (Millipore, USA) according to the manufacturer's instructions.

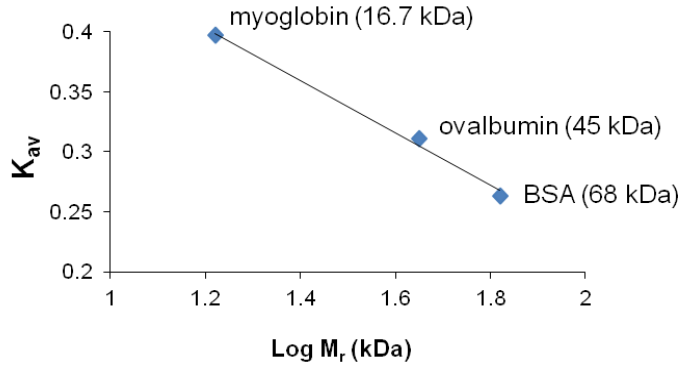

Figure 2.4 Fischer's plot for the estimation of protein M_r from MEC data. The Sephacryl S300 HR column was calibrated by applying a calibration sample in MEC buffer (25 mL/h, RT). The availability constant (K_{av}) was determined for each protein standard. The equation of the trend line is y = -0.2178x + 0.664
with a correlation co-efficient of 0.993.

2.3.4 Production of antibodies against VP2 peptides and recombinant VP2

2.3.4.1Selection and synthesis of VP2 peptides

The VP2 amino acid sequence was analysed using the Predict7™ software program which analyses protein structure according to the following parameters: hydrophilicity, surface probability, flexibility and antigenicity (Cármenes *et al.*, 1989). Two peptide sequences which demonstrated high hydrophilicity, surface probability, antigenicity and flexibility were selected (Table 2.2). The VP2 crystal structure (Lee *et al.*, 2006a) was also used to determine the location of the selected peptides to determine if they would be on the surface of the protein. The two peptides were modified before synthesis by the addition of an extra cysteine residue to the N- or C-terminus, to allow for coupling via M-maleimidobenzoil acid N-hydroxy succinimide ester (MBS) to rabbit albumin, the carrier protein. All internal cysteine residues were replaced with α-aminobutyric acid to avoid coupling of carrier protein through the internal cysteines. Peptides were synthesised by GL Biochem (Shanghai, China) at purity greater than 75%.

2.3.4.2Coupling of VP2 peptides to rabbit albumin

The synthesised peptides were prepared for immunisation by coupling to rabbit albumin via MBS that links the SH-group of a cysteine side chain to the ε-amino group of a lysine residue on the carrier protein. The coupling was carried out at a peptide:carrier ratio of 40:1 (Briand *et al.*, 1985). The MBS was allowed to react with rabbit albumin by mixing 5.26 mg rabbit albumin dissolved in 500 µL PBS with 0.97 mg MBS dissolved in 200 µL dimethyl formamide (DMF) and incubated with stirring for 30 min at RT before applying to a Sephadex® G-25 column (15 x 130 mm, 10 mL/h). The MBS-activated rabbit albumin was eluted with buffer A [100 mM sodium phosphate buffer pH 7.0, 0.02% (w/v) NaN_3] in 0.5 mL fractions. Eluted fractions were monitored by the A_{280} readings and all fractions with absorbance readings greater than 0.5 were pooled.

To reduce the peptides for coupling, the peptide (4 mg) was dissolved in 50 µL dimethyl sulfoxide (DMSO) and made up to 500 µL with peptide buffer [100 mM Tris-HCl buffer pH 7.0, 1 mM Na_2-EDTA, 0.02% (w/v) NaN_3]. The volume was made up to 1 mL with 10 mM DTT as reducing agent and incubated for 1 h at 37°C. The reduced peptide was separated from the reducing agent and non-reduced peptide

on a Sephadex® G-10 column (15 x 110 mm, 10 mL/h) using MEC buffer (0.15 M NaH_2PO_4, 0.05 M NaCl, pH 7.0) in 0.5 mL fractions. Elution fractions were analysed for reduced peptides by adding 10 µL Ellman's reagent [10 mM 5'5 dithiobis (2-nitro-benzoic acid), 100 mM Tris-Cl buffer pH 8.0, 10 mM Na_2-EDTA, 0.1% (w/v) SDS] to 10 µL of the elution fractions. Fractions containing reduced peptides turned yellow and could be distinguished from the second elution peak containing DTT that turned bright yellow. The reduced peptide containing fractions were pooled and mixed with the pooled MBS-activated rabbit albumin and incubated for 3 h at RT with stirring. The solution was equally aliquoted into four microfuge tubes and stored at -20°C until immunisation.

2.3.4.3 Immunisation of chickens with carrier-conjugated VP2 peptides and recombinant VP2

Hy-Line Brown hens aged between 25 to 40 weeks were used for immunisations. Animal ethics clearance was obtained from the UKZN Animal Research Ethics Committee (reference number 36/11/Animal). Purified VP2 expressed in *E. coli* (50 µg) (Section 2.3.2.3) and rabbit albumin coupled peptide (~200 µg) were separately triturated with Freund's complete adjuvant in a 1:1 (v/v) ratio to form a stable water-in-oil emulsion. Two chickens per antigen were immunised intramuscularly in each breast muscle. Booster immunisations were prepared by triturating purified VP2 (50 µg) or 200 µg rabbit albumin coupled peptide with Freund's incomplete adjuvant in a 1:1 (v/v) ratio. The booster immunisations were administered at week 2, 4 and 6 after the initial immunisation. Eggs were collected from all immunised chickens daily up to and including week 13. In addition, eggs were collected prior to the initial immunisation for the isolation of pre-immune antibodies.

2.3.4.4 Isolation of chicken anti-VP2 peptide and chicken anti-VP2 antibodies

Antibodies were isolated from chicken egg yolk according to Polson *et al.* (1985) with minor modifications (Goldring and Coetzer, 2003). Briefly, the egg yolk was separated from the egg white and rinsed under running tap water to remove excess egg white. The yolk was mixed with two volumes of IgY isolation buffer [100 mM Na-phosphate buffer, pH 7.6, 0.02% (w/v) NaN_3] after which 3.5% (w/v) PEG M_r 6000 was added and dissolved with stirring. The sample was centrifuged (4420 x *g,* 20 min, RT) and the supernatant filtered through absorbent cotton wool. The filtrate volume was measured

and 8.5% (w/v) PEG M_r 6000 added and dissolved by stirring before centrifugation (12000 x *g*, 10 min, RT.) The supernatant was discarded and the pellet was dissolved in isolation buffer in a volume equal to the original egg yolk volume. To the dissolved pellet, 12% (w/v) PEG 6000 was added and dissolved by stirring before centrifugation (12 000 x *g,* 10 min, RT). The supernatant was discarded and the final pellet was resuspended in 1/6 of the original egg yolk volume of isolation buffer. Antibody concentration was determined by measuring A_{280} and using the extinction coefficient $E_{280\ nm}^{1\ mg/ml}$=1.25 (Goldring and Coetzer, 2003).

2.3.4.5Preparation of affinity matrices for antibody purification

The VP2 peptides and purified VP2 expressed in *P. pastoris* were each coupled to affinity matrices in order to purify the chicken anti-VP2 peptide IgY and the chicken anti-VP2 IgY respectively. Coupling was carried out under gravitational flow-rate. Peptides were coupled to SulfoLink® Coupling Resin according to the manufacturer's instructions. Briefly, the 50% slurry of SulfoLink® coupling resin (2 mL) was transferred to a Poly-Prep® Chromatography column and allowed to settle at RT. The storage buffer was drained and the column was equilibrated with four column volumes of coupling buffer (50 mM Tris-HCl, 5 mM EDTA-Na; pH 8.5). The peptide was reduced as before (Section 2.3.4.2) prior to coupling to the SulfoLink® coupling resin, made up to 2 mL with coupling buffer and incubated with the SulfoLink® coupling resin on an end-over-end rotator for 15 min at RT. The resin was allowed to stand and incubate for a further 30 min before draining and washing of the column with three column volumes of coupling buffer. The non-specific binding sites were blocked by incubating the resin with one column volume of 50 mM L-cysteine-HCl in coupling buffer on an end-over-end rotator for 15 min at RT and a further 30 min in an upright position. The column was washed with six column volumes of 1 M NaCl before washing in storage buffer in which it was stored.

Purified VP2 (5 mg) was coupled to AminoLink® Plus Resin according to the manufacturer's instructions. Briefly, the 50% resin slurry (2 mL) was transferred to a Poly-Prep® Chromatography column and allowed to settle at RT. The storage buffer was drained and the resin bed equilibrated with three column volumes of coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2). The VP2 sample was made up to 6 mL with coupling buffer and the reductive amidation of the primary Schiff's bases carried out with $NaCNBH_3$ (50 mM final concentration) before incubation with the

resin overnight on an end-over-end rotator at 4°C. The contents were drained and the resin washed with 2 mL coupling buffer. The remaining sites were blocked by washing the resin with 2 mL quenching buffer (1 M Tris-HCl, pH 7.4) before a final round of reductive amidation of the Schiff's bases with $NaCNBH_3$ (50 mM final concentration) in 1 mL quenching buffer and incubation on an end-over-end rotator for 30 min at RT. The contents were drained and the column was washed with five column volumes of 1 M NaCl followed by washing with 3 mL storage solution (0.1 M sodium phosphate, 0.15 M NaCl, 0.05% (w/v) NaN_3, pH 7.2) in which it was stored.

2.3.4.6 Affinity purification of chicken anti-VP2 peptide and chicken anti-VP2 antibodies

The isolated chicken anti-VP2 peptide and chicken anti-VP2 antibodies (Section 2.3.4.4) were pooled and affinity purified on their respective affinity columns. Antibodies (60 mL) were filtered through Whatman no. 1 filter paper and circulated overnight in a reverse direction over the respective affinity matrices at RT. The column was washed with twenty column volumes of wash buffer [100 mM sodium phosphate buffer, 0.02% (w/v) NaN_3, pH 6.5] and fractions (900 µL) eluted with elution buffer (100 mM glycine-HCl, pH 2.8) into a tube containing 100 µL neutralisation buffer (1 M sodium phosphate buffer, pH 8.5). Elution fractions were monitored by measuring the A_{280} values and all fractions with absorbance values >0.4 were pooled and stored at 4°C after the addition of NaN_3 to a final concentration of 0.1% (w/v).

2.3.4.7 Indirect enzyme–linked immunosorbent assay (ELISA) to monitor antibody production

An ELISA was used to monitor the production of chicken anti-VP2 peptide and chicken anti-VP2 antibodies over a 13 week period after the first immunisation and to determine the level of recognition of affinity purified antibodies. Antibody titres were monitored by coating Nunc 96 microtiter plate wells overnight at 4°C with 150 µL of 1 µg/mL or 5 µg/mL peptide or recombinant VP2 in PBS. All unoccupied sites in the wells were blocked by incubating with 200 µL blocking solution [0.5% (w/v) BSA in PBS] for 1 h at 37°C and washed three times with 0.1% (v/v) PBS-Tween 20. Antibody titres from each week were determined by diluting antibodies in blocking solution from 250 µg/mL to 0.001 µg/mL and incubating in the wells (100 µL) in duplicate for 2 h at 37°C. Pre-immune, non-affinity purified and unbound antibodies were included as

controls to determine antibody levels of recognition. The plate was washed as before prior to the addition of 120 µl rabbit anti-IgY-HRPO conjugate (1:12 000 dilution) and incubated for 1 h at 37°C. The secondary antibody was removed and the plate washed as before. The chromogen/substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in 0.15 M citrate-phosphate buffer, pH 5.0] (150 µL) was added to the wells and allowed to react in the dark for 30 - 45 min before reading the absorbance values at 405 nm using a FLUORStar Optima spectrophotometer (BMG Labtech, Germany).

2.3.5 Analytical Biochemical Methods

2.3.5.1Agarose gel electrophoresis

Agarose [1% (w/v)] was dissolved in TAE buffer (200 mM Tris-acetate buffer, pH 8.0, 5 mM Na_2-EDTA) by gentle heating. The gel solution was cooled down until the glass flask could be physically touched (±55°C) after which ethidium bromide was added to a final concentration of 0.5 µg/mL. Loading buffer [10 mM Tris-HCI buffer pH 8, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF, 60 mM EDTA, 60% (v/v) glycerol] was added to all samples in a 5:1 sample/loading buffer ratio. Electrophoresis of all DNA samples was performed at 60-100 V for 45 min and images were captured with a VersaDoc™ imaging system (BioRad, Hercules, USA) under UV light.

2.3.5.2Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE analysis was performed according to LaemmLi (1970) using the Bio-Rad Mini Protean III® vertical slab electrophoresis apparatus. Protein samples which were analysed under reducing conditions were prepared by adding an equal volume of reducing treatment buffer [125 mM Tris-HCI buffer, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 6.8] to the protein sample, vortexing and boiling for 5 min. Protein samples analysed under non-reducing conditions were prepared by adding half the total volume of non-reducing treatment buffer [125 mM Tris-HCI buffer, 4% (w/v) SDS, 20% (v/v) glycerol, pH 6.8] to the protein sample. Before loading samples on the gel, 25% (w/v) bromophenol blue was added (5 µL). Samples were subjected to electrophoresis at 18 mA per gel until the bromophenol blue tracker dye was about 0.5 cm from the bottom edge of the gel. The molecular mass marker sample was made up of phosphorylase b (97 kDa), BSA (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). The marker was prepared by making up 5 mg/mL protein stocks

with treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, pH 6.8] and combining 80 µL of each of the protein stocks together with 20 µL reducing treatment buffer [10% (v/v) 2-mercaptoethanol in treatment buffer] and 25% (w/v) bromophenol blue. The marker was boiled for 2.5 min and 4 µL loaded per gel. Since the relative mobility of a protein is inversely proportional to its log molecular mass, a calibration curve was generated from the known protein molecular masses to determine unknown protein sizes (Figure 2.5). A PageRuler™ Pre-stained Protein Ladder (Fermentas, Luthuania) was also used.

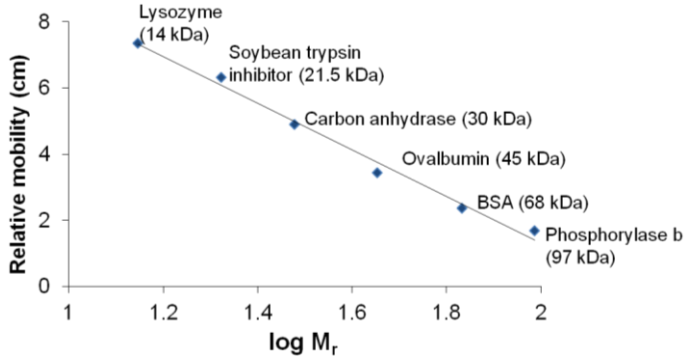

Figure 2.5 Calibration curve for determining unknown protein molecular masses. The molecular mass proteins: phosphorylase b (97 kDa), BSA (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa), were separated by 10% reducing SDS-PAGE and their relative mobility plotted against their log molecular weight. The equation of the trend line is y = -7.062x + 15.424 and the correlation co-efficient is 0.9899.

Silver staining

Protein bands were visualised by staining with silver nitrate, Coomassie Blue R-250 or Oriole™ Fluorescent Gel Stain (Bio-Rad, USA). The silver nitrate staining method was carried according to Blum *et al.* (1987) on an orbital shaker at RT using thoroughly cleaned glassware. Gels were immersed in fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) 37% formaldehyde] for 1 hour or overnight and placed in washing solution [50% (v/v) ethanol] (3 × 20 min). Gels were incubated for 1 min in pre-treatment solution [0.02% (w/v) sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$) and rinsed in distilled water (3 × 5 min) before soaking in impregnation solution [0.2% (w/v) silver nitrate, 0.75% (v/v) 37% formaldehyde] for 20 min. The gel was rinsed in distilled water (2 × 20 s) and incubated in developing solution [60 g/L Na_2CO_3, 0.5% (v/v) 37% formaldehyde, 0.004% (w/v) $Na_2S_2O_3.5H_2O$] until the first protein bands became visible. Development was allowed to proceed in distilled water and was stopped by

immersing the gel in stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] for 10 min. The gel was transferred to 50% (v/v) methanol before the image was captured with the VersaDoc™ imaging system (Bio-Rad, USA).

Coomassie Brilliant Blue R-250 stain

For the Coomassie R-250 stain, gels were placed in Coomassie stain [0.125% (w/v) Coomassie Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid], destained in Destain I [50% (v/v) methanol, 10% (v/v) acetic acid] and Destain II [7% (v/v), 5% (v/v)]. Gels were imaged using the Bio-Rad VersaDoc™ imaging system.

Oriole™ Fluorescent Gel Stain

Gels were also stained with the Oriole™ Fluorescent Gel Stain (BioRad, USA) according to the manufacturers' instructions. The Oriole™ stain can detect purified proteins at extremely low concentrations of approximately 0.001-0.002 µg.

2.3.5.3Western blotting

Proteins were electro-transferred onto nitrocellulose membrane for western blotting after separation by SDS-PAGE using a wet blotter (BioRad, Hercules, USA). This was done by sandwiching the gel and nitrocellulose membrane between two sets of blotting paper and one set of sponges in a blotting cassette. The gel, nitrocellulose membrane and blotting paper were soaked in blotting buffer [20 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, 1% (w/v) SDS] prior to transfer which was conducted at 40 mA for 16 h. The membrane was transiently stained for 5 min with Ponceau S stain [0.1% (w/v) Ponceau S, 1% (v/v) glacial acetic acid] to determine the quality of protein transfer and to mark the molecular weight marker positions with a pencil (if an unstained marker was used). The membrane was destained in distilled water containing a few drops 1 M NaOH solution. The nonspecific sites on the nitrocellulose membrane were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) (20 mM Tris, 200 mM NaCl, pH 7.4) for 1 h with gentle shaking. Thereafter the membrane was washed with TBS (3 x 5 min) and incubated for 2 h with primary antibody [1:2 000 mouse anti-His tag monoclonal antibody or 100 µg/mL chicken anti-VP2 peptide or chicken anti-VP2 antibodies] made up in 0.5% (w/v) BSA-TBS and washed as before in TBS (3 x 5 min). Following washing,

the membrane was incubated in secondary antibody [horse anti-mouse-HRPO (1:12 000) or rabbit anti-chicken-HRPO (1:12 000)] in 0.5% (w/v) BSA-TBS for 1 h and again washed in TBS (3 x 5 min). Finally the nitrocellulose membrane was immersed in chromogen/substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, 0.0015% (v/v) H_2O_2 in TBS], allowed to develop in the dark and placed in distilled water once protein bands were clearly visible.

2.3.5.4Quantification of purified protein

Purified VP2 samples were quantified using the BCA™ Protein Assay kit (Pierce, USA) according to the manufacturer's instructions in a Nunc™ 96 microtiter plate. The absorbance readings (A_{562}) of standard BSA samples and samples of unknown protein concentration were determined using a FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany) and used to generate a standard curve for the quantification of protein (Figure 2.6). Alternatively the quantification was carried out by running the BSA standard samples on reducing SDS-PAGE alongside the protein samples to be determined.

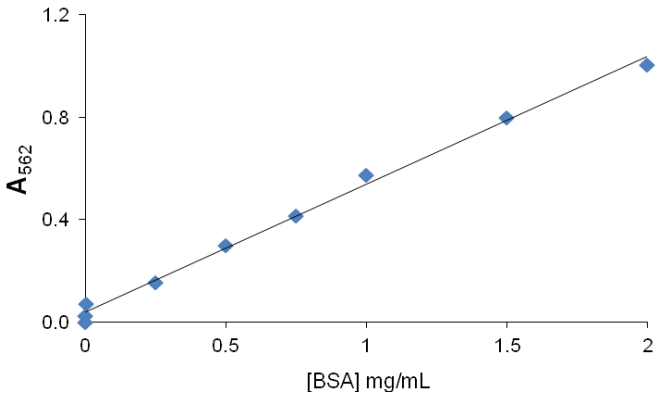

Figure 2.6 Standard curve for the quantification of proteins. The BCA™ Protein Assay Kit was used to generate the curve using BSA as protein standards ranging from 0.125 – 2.0 mg/mL. The equation of the trend line is y = 0.4989x + 0.0381 and the correlation coefficient is 0.9949.

2.4 RESULTS

2.4.1 Bacterial Expression of VP2

2.4.1.1Expression of VP2 using pGEX-4T-1

Expression of VP2 in pGEX-4T-1 was attempted in 2xYT broth by IPTG induction. Genes in pGEX-4T-1 are expressed as a protein with a GST-tag which has a

molecular mass of 26 kDa. The VP2 protein has a molecular mass of 47 kDa therefore the expression of VP2 in pGEX-4T-1 is expected to yield a fusion protein with a molecular mass of 73 kDa. The *E. coli* cell lysates (20 µg/well) were analysed for VP2 expression by SDS-PAGE (Figure 2.7). Although uninduced lysate (lane 1) show a similar profile as the induced lysate (lane 2) the band intensity in the uninduced lysates was lower than the induced sample. Expression of VP2 was therefore unsuccessful in this system even after optimisation of expression conditions such as IPTG concentration, expression temperature and time. Western blotting using chicken anti-GST tag antibodies confirmed the lack of expression when this system was used (result not shown).

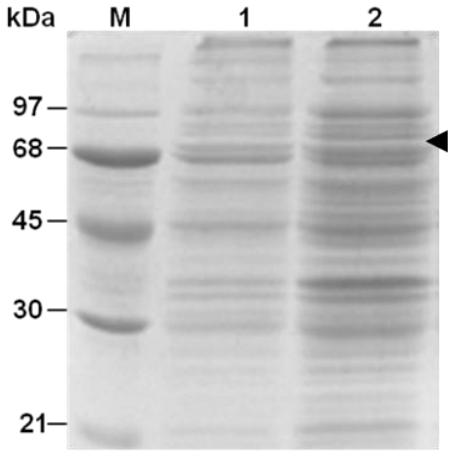

Figure 2.7 Expression analysis of pGEX-4T-1-VP2 construct on a 10% reducing SDS-PAGE stained with Coomassie blue R-250. Lane M, molecular weight marker; lane 1, uninduced pGEX-4T-1-VP2 lysate and lane 2, induced pGEX-4T-1-VP2 lysate. Arrow indicates M_r at which VP2 is expected.

2.4.1.2Expression of VP2 using pET-32a

Expression of the pET-32a recombinants was performed by both auto-induction in Terrific broth (Figure 2.8A) and IPTG induction in 2xYT medium (Figure 2.8B). Genes cloned in the pET-32a system are expressed with an N-terminal Trx-His tag which has a molecular mass of 17 kDa. Therefore the expected size for the recombinant fusion protein expressed in this system was 64 kDa. The *E. coli* BL21(DE3) cells were used as a control for the expression of VP2 in Terrific broth (Figure 2.8A, lane 1). The His-tagged VP2 was successfully expressed as a 64 kDa fusion protein (Figure 2.8A, lane 2) as expected. High molecular mass proteins at the top of the gel were also observed in the lysate. For the IPTG-induced expression, a non-induced lysate was used as a control (Figure 2.8B, lane 1). The

IPTG induced expression was unsuccessful as the induced lysate demonstrated no expression of a 64 kDa VP2 protein (Figure 2.8B, lane 2). In addition, western blotting was used to confirm that the 64 kDa protein band observed in the auto-induced expression was the recombinant Trx-His-VP2 fusion protein (Figure 2.8C). Due to the unavailability of chicken anti-VP2 antibodies at the time, mouse anti-His tag antibodies were used. The mouse anti-His tag antibodies did not detect any proteins in the control lysate (Figure 2.8C, lane 1), but recognised the 64 kDa protein band corresponding to VP2 fusion protein as well as high molecular mass proteins (Figure 2.8C, lane 2).

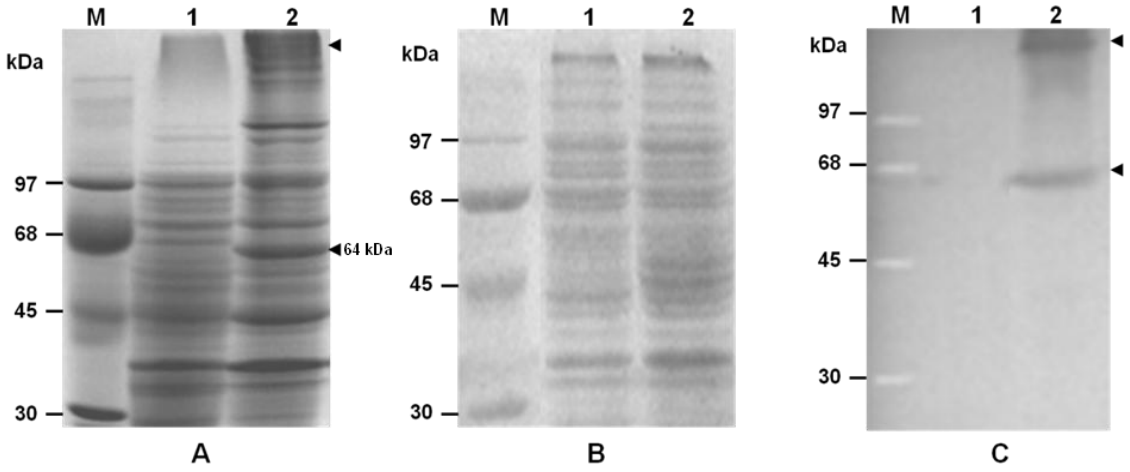

Figure 2.8 Analysis of VP2 expression using the pET-32a vector in *E. coli* BL21(DE3) cells. (A) 10% reducing SDS-PAGE analysis of VP2 auto-induced expression in Terrific broth. **(B)** 10% reducing SDS-PAGE analysis of VP2 IPTG induced expression. **(C)** Western blot analysis of VP2 auto-induced expression detected with a monoclonal mouse anti-His tag antibody. Lane M, molecular weight marker; lane 1, *E. coli* BL21(DE3) control lysate (A and C) and non-induced lysate (B) and lane 2, auto-induced pET-32a-VP2 lysate (A and C) and IPTG induced lysate (B). Arrows indicate expressed proteins.

The solubility of the expressed VP2 sample was determined by separating the bacterial cell pellet (insoluble fraction, I) and supernatant (soluble fraction, S). When analysed on reducing SDS-PAGE, the band corresponding to the fusion protein was only observed in the insoluble fraction suggesting that the protein was expressed as inclusion bodies (Figure 2.9A). In an attempt to improve the solubility of VP2, the expression was carried out at different temperatures below 37°C (Figure 2.9B). The VP2 expression at 16°C and 25°C was negligible with a small degree of VP2 visible only in the insoluble fraction (Figure 2.9B). Even though the expression level was high at 30°C, the fusion protein was still in the insoluble fraction (Figure 2.9B). Changing the temperature conditions did not improve the solubility of VP2, therefore VP2 expression was continued at 37°C.

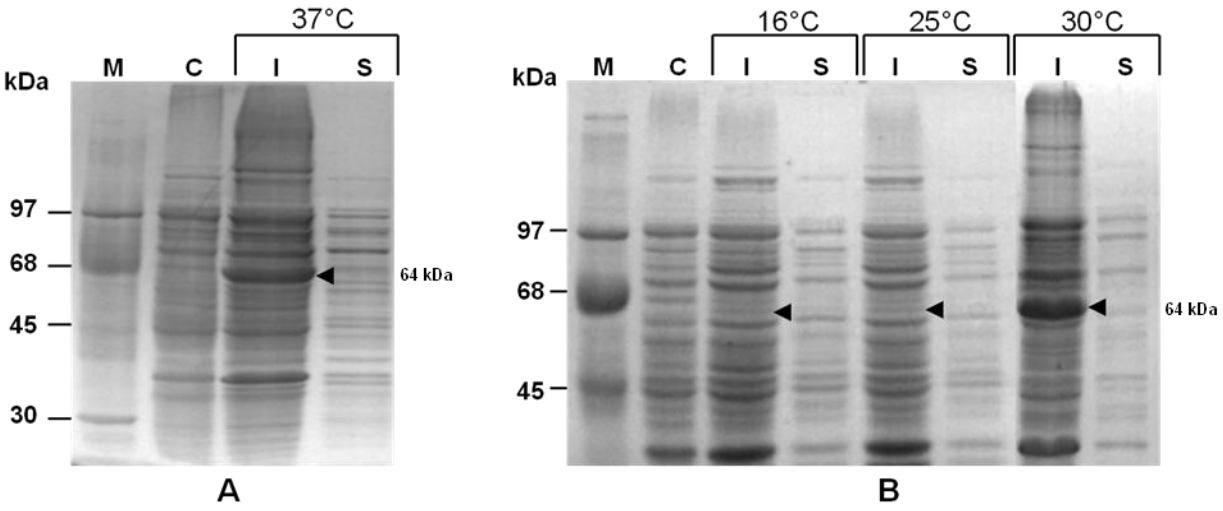

Figure 2.9 Analysis of VP2 solubility after expression at different temperatures analysed by reducing 10% SDS-PAGE stained with Coomassie Blue R-250. Expression was performed at 37°C **(A)** 16°C, 25°C and 30°C **(B)**. Key: molecular weight marker (M), *E. coli* BL21(DE3) control cell lysate (C); soluble (S) and insoluble (I) fractions. Arrows indicate VP2.

The inclusion bodies were subjected to three washes in 2 M urea prior to solubilisation in 8 M urea (Figure 2.10). Some of the VP2 protein was released during the first two washes (lanes 2 and 3), but not in the last wash (lane 4). The washed inclusion bodies were solubilised in 8 M urea and thereafter centrifuged to separate the insoluble aggregates (lane 5) and solubilised protein (lane 6). Recombinant VP2 was successfully solubilised as shown by the presence of the 64 kDa fusion protein in the solubilisation supernatant.

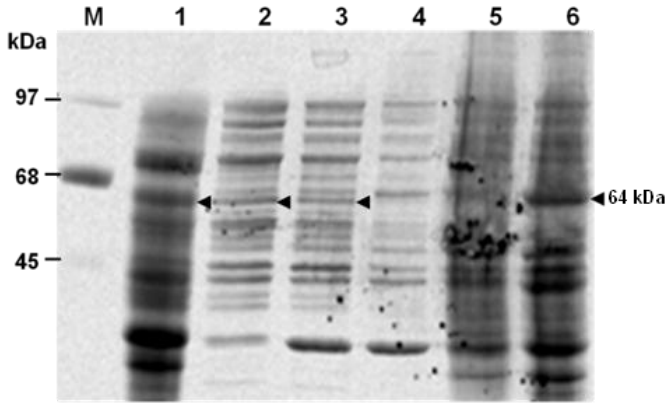

Figure 2.10 Reducing SDS-PAGE (10%) analysis of VP2 solubilisation stained with Coomassie blue R-250. Lane M, molecular mass marker; lane 1, VP2 insoluble fraction; lanes 2 to 4, 2 M urea washes; lane 5, insoluble aggregates after 8 M urea solubilisation and lane 6, solubilised proteins after 8 M urea solubilisation. Arrows indicate VP2 protein.

2.4.2 Purification of VP2 expressed in *E. coli*

2.4.2.1Affinity purification of insoluble Trx-His tagged recombinant VP2

The solubilised sample containing VP2 was purified on a Ni-NTA His-Bind affinity column under denaturing conditions and the purification steps were analysed by reducing SDS-PAGE and western blotting (Figure 2.11). An *E. coli* BL21(DE3) cell lysate (lane 1) and a VP2 solubilised fraction (lane 2) were separated alongside the purification fractions for comparison. The VP2 bound poorly to the affinity matrix as evidenced by the presence of VP2 in the unbound fraction (Figure 2.11A, lane 3). Proteins, including some VP2, were also released during the washing step (Figure 2.11A, lane 4) although a considerable amount of VP2 was released during elution with imidazole, with co-purification of bacterial proteins (Figure 2.11A, lanes 5-7). In the western blot analysis, mouse anti-His tag antibodies did not detect any proteins in the *E. coli* BL21(DE3) cell lysate control (Figure 2.11B, lane 1). The VP2 protein at the expected size of 64 kDa and high molecular mass proteins were detected in the solubilised, unbound and wash fractions (Figure 2.11B, lanes 2-4). The VP2 purification under denaturing conditions was considered an unfavourable method of purification because eluted proteins are in a denatured state and require further steps to refold the protein.

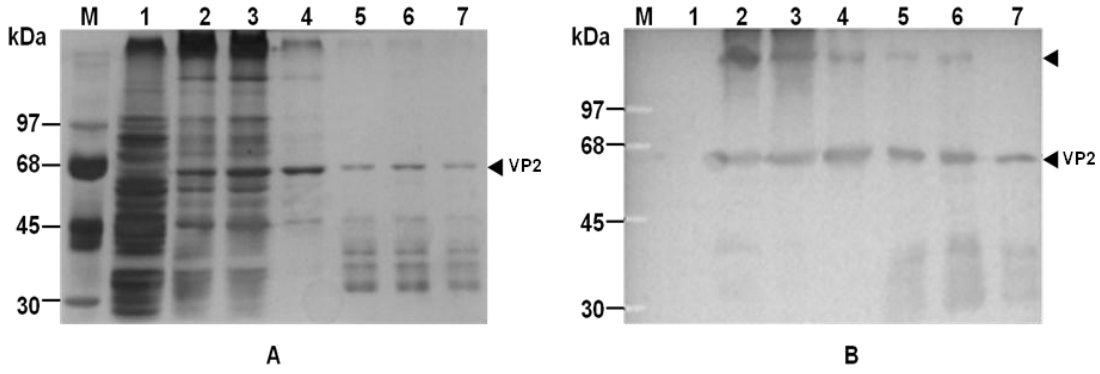

Figure 2.11 Analysis of VP2 purification under denaturing conditions on a Ni-NTA His-Bind matrix. (A) Reducing 10% SDS-PAGE stained with Coomassie Blue R-250. **(B)** Western blot using a monoclonal mouse anti-His tag antibody. Lane 1, *E. coli* BL21 (DE3) control lysate; lane 2, solubilised VP2; lane 3, unbound fraction; lane 4, wash fraction, lanes 5-7, VP2 elution fractions. Arrows indicate VP2 and high molecular mass proteins.

To obtain correctly folded VP2 for subsequent use, the solubilised sample containing VP2, was refolded on a Ni-NTA His-Bind affinity column. Denatured VP2 was subjected to refolding during the washing step by using a descending concentration gradient from 8 M urea to 0 M urea. Eluted fractions were analysed by reducing SDS-PAGE (Figure 2.12A) and western blotting (Figure 2.12B). An *E. coli*

BL21 (DE3) cell control lysate (Figure 2.12A, lane 1) and VP2 expression sample (Figure 2.12A, lane 2) were analysed alongside the purified refolded VP2 fractions (Figure 2.12A, lanes 3-8). In the western blot the mouse anti-His tag antibody detected the fusion protein in the expression sample (Figure 2.12B, lane 2), unbound fraction (Figure 2.12B, lane 3), wash fraction (Figure 2.12B, lane 4) and the refolded sample (Figure 2.12B, lane 5). High molecular mass proteins were also recognised by the monoclonal mouse anti-His tag antibody (Figure 2.12B, lane 2) and in the refolded VP2 fractions (Figure 2.12B, lanes 5 and 6). The non-reduced refolded VP2 fraction (Figure 2.12B, lane 6) was detected at a size larger than the reduced refolded fraction which indicates the protein was successfully refolded.

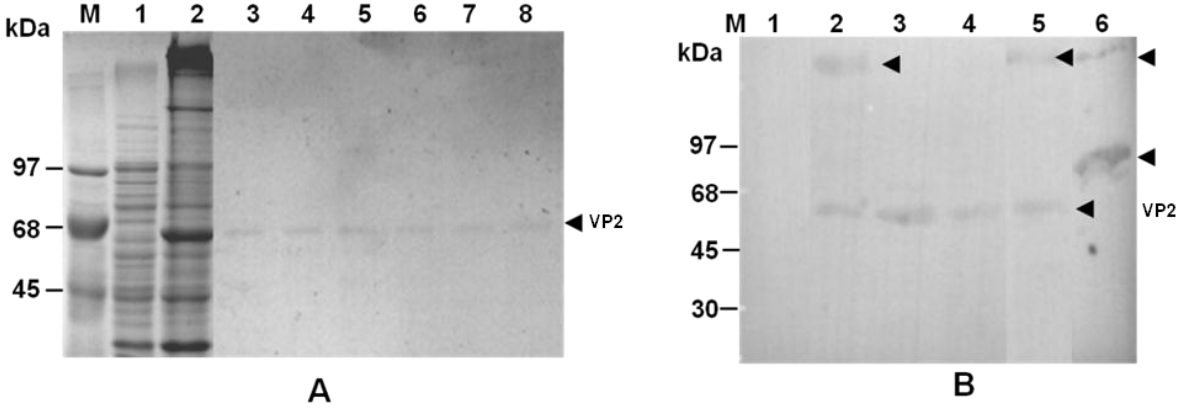

Figure 2.12 Analysis of VP2 on-column refolding purification using Ni-NTA His-Bind resin. (A) 10% reducing SDS-PAGE stained with silver nitrate. Lane M, molecular weight marker; lane 1, *E. coli* BL21(DE3) cell control lysate; lane 2, VP2 expression sample; lanes 3 to 8, VP2 purified refolded fractions. **(B)** Western blot using mouse anti-His tag antibodies. Lane M, molecular weight marker; lane 1, *E. coli* BL21(DE3) cell control lysate; lane 2, VP2 expression sample; lane 3, unbound fraction; lane 4, wash fraction; lane 5, reduced refolded VP2 and lane 6, non-reduced refolded VP2. Arrows indicate VP2 and high molecular mass proteins.

2.4.2.2Cation-exchange chromatography of VP2 wash fractions

Ion-exchange chromatography is occasionally used as a secondary purification step following affinity chromatography to obtain pure proteins for protein crystallisation (Dennison, 2003) or for further purification of antibodies (Liu *et al.*, 2010). Since most of the VP2 protein was released during the washing step with minimal contaminating proteins during purification under denaturing conditions, the wash fractions were retained and dialysed in two changes of IEC buffer for further purification using cation exchange chromatography. Protein elution was observed after the addition of 0.5 M NaCl as shown by higher A_{280} readings of the fractions (Figure 2.13A). The addition of 0.05 M NaCl and 0.1 M NaCl appears to release low

quantities of the protein as indicated by the low A_{280} readings. Various elution fractions from the peaks observed were further analysed by SDS-PAGE (Figure 2.13B) as indicated by the arrows below the elution profile.

The eluted fractions were analysed along with the dialysed VP2 sample (lane 1). Fraction 13 (lane 2) and 15 (lane 3) from the first peak contained some VP2 at the expected size of 64 kDa, as well as contaminating proteins and high molecular mass proteins. Fractions 25 (lane 4) and 27 (lane 5) analysed after the addition of 0.05 M NaCl showed a light protein band above 68 kDa. Fractions 37 (lane 6) and 39 (lane 7) eluted after the addition of 0.1 M NaCl, contained VP2, some high molecular mass proteins and other contaminating proteins around 30 kDa. Fraction 57 (lane 8) and 59 (lane 9) from the second peak analysed after the addition of 0.5 M NaCl contained VP2 at the expected size of 64 kDa as well as some high molecular mass proteins.

2.4.2.3Electro-elution of VP2

In addition to the expression of the VP2 protein, high molecular mass proteins were also observed (Figure 2.8A and C) and co-purified with VP2 on a Ni-NTA His-Bind affinity column (Figure 2.11). The high molecular mass proteins were therefore hypothesised to be VP2 multimers or incorrectly folded VP2. To further identify this, both VP2 (64 kDa) and the high molecular mass proteins were excised from SDS-PAGE gels, electro-eluted and analysed by SDS-PAGE (Figure 2.14). An *E. coli* BL21(DE3) cell control lysate (lane 1) and expression sample (lane 2) were analysed alongside the electro-eluted VP2 sample (lane 3) and the high molecular mass electro-eluted sample (lane 4). The electro-eluted VP2 sample was observed at the expected size of 64 kDa (lane 3). The 64 kDa protein band was also observed when the high molecular mass protein was electro-eluted (lane 4). The electro-elution of the high molecular mass proteins was repeated several times with the same observable result on SDS-PAGE (lane 4). Since analysis of the high molecular mass proteins on SDS-PAGE reduced some of the proteins to a 64 kDa protein and mouse anti-His tag monoclonal antibodies used to detect VP2 expression recognised the high molecular mass proteins (Figure 2.8C, 2.11B and 2.12B), these proteins were believed to be VP2 multimers. Although the VP2 protein was successfully expressed in the *E. coli* expression system, purification of sufficient

quantities was difficult. Therefore the *P. pastoris* expression system was also used to increase the yield.

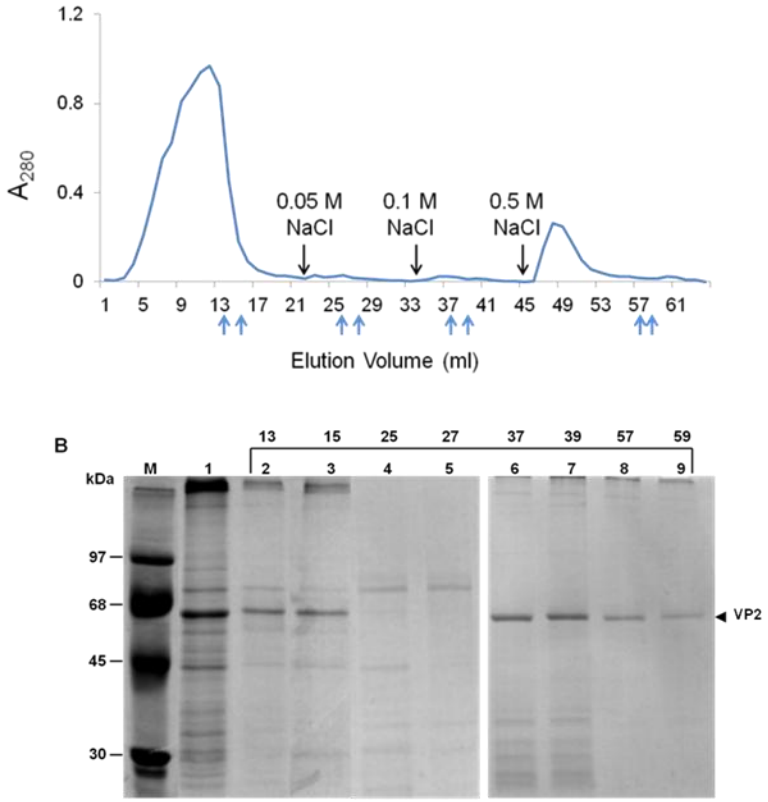

Figure 2.13 Analysis of IEC purification of Trx-His tagged recombinant VP2. (A) Elution profile of VP2 purification on SP-Sephadex C-25 cation exchange chromatography column (25 x 150 mm; 30 mL/h). A step-wise gradient of NaCl was applied at 23 mL (0.05 M), 35 mL (0.1 M) and 47 mL (0.5 M) elution volume. The arrows indicate the fractions used for SDS-PAGE analysis. **(B)** Analysis of IEC elution fractions by reducing 10% SDS-PAGE stained with silver nitrate. Lane M, molecular weight marker; lane 1, dialysed VP2 sample; lanes 2-9 contain the eluted fractions as indicated.

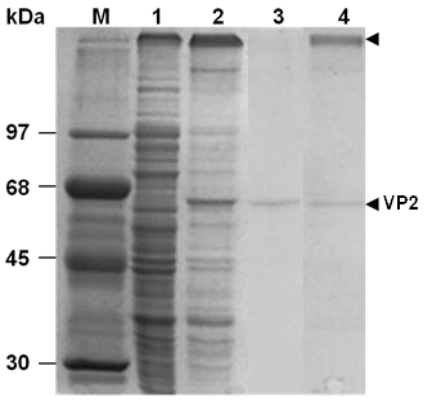

Figure 2.14 Analysis of the electro-eluted VP2 and high molecular mass proteins. Samples were separated on a 10% reducing SDS-PAGE gel and stained with silver nitrate. Lane M, molecular weight marker; lane 1, *E. coli* BL21(DE3) cell lysate; lane 2, VP2 expression sample; lane 3, electro-eluted VP2 sample; lane 4, electro-eluted high molecular mass sample. Arrows indicate electro-eluted proteins.

2.4.3 VP2 expression in *P. pastoris*

The VP2 coding region was previously cloned into the pGEX-4T-1 and pET-32a expression vector and transformed into *E. coli* JM109 BL21 and BL21(DE3) cells, respectively. The VP2 coding region from recombinant pGEX-4T-1 plasmid was used for sub-cloning into the pPIC9 vector. This was achieved by performing a DNA plasmid miniprep isolation and double restriction digest with EcoRI and NotI to release the VP2 insert DNA (Figure 2.15). The pPIC9 expression vector was also isolated (lane 3) and subjected to a double restriction digest with EcoRI and NotI to prepare the vector for ligation with the VP2 insert DNA. Uncut recombinant pGEX-4T-1 plasmid containing the VP2 gene was observed as a single band at approximately 5 kb (lane 1). An uncut plasmid usually runs as three bands which correspond to the three conformations of DNA namely, supercoiled, linear and circular DNA. However, in this study uncut DNA was only visible as a single band which could be the result of low DNA concentration. The double restriction digestion of recombinant pGEX-4T-1 with EcoRI and NotI released two bands corresponding to vector (5 kb) and insert DNA at 1.2 kb (lane 2). The purified linearised vector was observed at 8 kb as expected (lane 4).

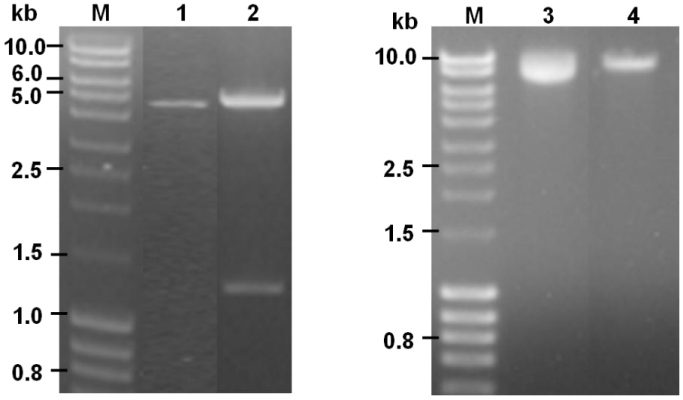

Figure 2.15 Analysis of plasmid DNA miniprep isolation and double restriction digestion of recombinant pGEX-4T-1-VP2 and non-recombinant pPIC9 expression vector. The recombinant pGEX-4T-1-VP2 and non-recombinant pPIC9 expression vector was restriction digested with EcoRI and Not I and analysed on a 1% (w/v) agarose gel stained with ethidium bromide. Lane M, MassRuler DNA ladder; lane 1, uncut pGEX-4T-1-VP2; lane 2, cut pGEX-4T-1-VP2; lane 3, uncut pPIC9 and lane 4, cut pPIC9.

The VP2 insert DNA was ligated to the pPIC9 expression vector and transformed into *E. coli* JM109 cells. Colonies were screened for recombinants by colony PCR using AOX vector primers (Figure 2.16). The AOX primers are expected to amplify a 490 bp fragment for a non-recombinant plasmid, therefore for a recombinant

plasmid, the amplicon size should be 490 bp plus the size of the VP2 insert DNA, i.e. 1.2 kb. Of the two colonies analysed, both were recombinant for the 1.2 kb VP2 gene as seen by the amplification of a 1.7 kb fragment (lanes 1 and 2).

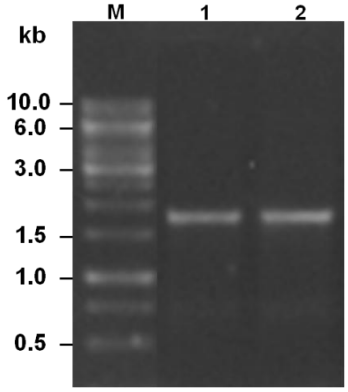

Figure 2.16 Analysis of colony PCR screening of pPIC9-VP2 recombinants. Colonies were screened for recombinants using AOX vector primers and analysed on a 1% (w/v) agarose gel stained with ethidium bromide. Lane M, MassRuler DNA ladder; lanes 1 and 2, colonies 1 and 2.

A DNA miniprep isolation was performed on the *E. coli* cells containing the pPIC9-VP2 recombinant plasmid and linearised with SacI in preparation to be integrated into the *P. pastoris* genome (Figure 2.17). The DNA isolation (lane 1) and linearised DNA (lane 2) was observed at the expected size of approximately 10 kb.

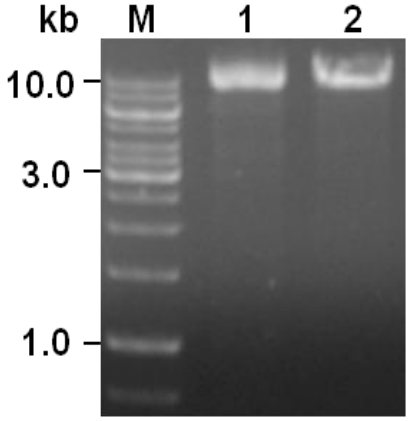

Figure 2.17 Analysis of SacI digestion of pPIC9-VP2 recombinant plasmid. The restriction digested pPIC9-VP2 recombinant was analysed on a 1% (w/v) agarose gels stained with ethidium bromide. Lane M, MassRuler DNA ladder; lane 1, uncut pPIC9-VP2 and lane 2, cut pPIC9-VP2 linearised with SacI.

The linearised pPIC9-VP2 recombinant plasmid was transformed into *P. pastoris* GS115 yeast cells and screened for recombinants by colony PCR using the universal AOX primers (Figure 2.18). Of the two colonies that grew, one gave a DNA

fragment of approximately 500 bp (lane 1) whereas a size of 1.9 kb is expected for a successful clone. A DNA fragment could not be clearly observed for the second colony (lane 2) and the PCR therefore gave inconclusive results.

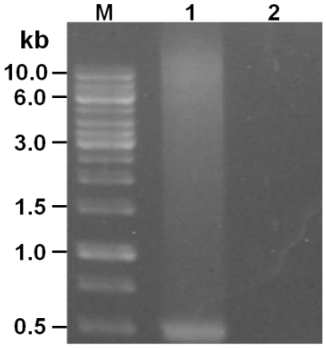

Figure 2.18 Colony PCR of *P. pastoris* GS115 cells transformed with SacI-linearised recombinant pPIC9 plasmids containing VP2. In order to determine if integration into the genomic DNA was successful, samples were analysed on a 1% (w/v) agarose gel and stained with ethidium bromide. Lane M, MassRuler DNA ladder; lane 1 and 2, PCR products after amplification with AOX primers of colonies 1 (lane 1) and 2 (lane 2).

Although the PCR results were inconclusive to determine whether pPIC9-VP2 was successfully integrated into the *P. pastoris* genome, a decision was made to continue with expression to determine the success of the transformation. Therefore, the colony was used to inoculate growth medium and induced for expression by daily supplementation with methanol. Protein expression was analysed by SDS-PAGE and visualised with silver nitrate staining (Figure 2.19). The VP2 was successfully expressed at the expected size of 47 kDa along with high molecular mass proteins and an 80 kDa protein (lane 2).

Three-phase partitioning was used as an initial concentrating and purification step after VP2 expression in the *P. pastoris* system (Figure 2.19). The TPP method uses t-butanol and ammonium sulfate as a salting-out type fractionation method which is useful for precipitating out proteins in solution. Proteins in the induced supernatant were efficiently precipitated using TPP as indicated by the prominent smear of high molecular mass proteins (lane 3).

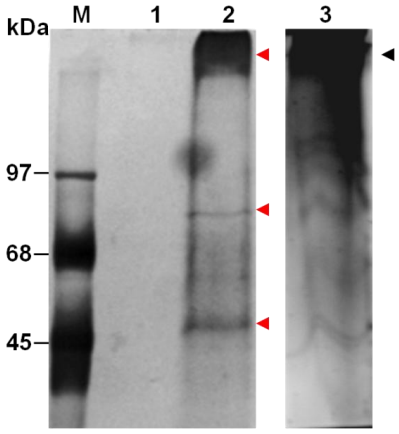

Figure 2.19 Analysis of VP2 expression in P. pastoris following concentration of the expressed supernatant by TPP. Samples were separated by reducing 10% SDS-PAGE and stained with silver nitrate. Lane M, molecular weight marker; lane 1, non-induced expression supernatant; lane 2, induced expression supernatant; lane 3, expression supernatant concentrated using TPP. Red arrows indicate expressed proteins and the black arrow indicates VP2 after TPP.

In order to purify VP2 from other contaminating proteins, MEC using a Sephacryl S300 chromatography column which has a fractionation range of 10 to 1500 kDa (Dennison, 1999) was used. The resin sufficiently separated the calibration proteins into four distinct peaks (Figure 2.20A) and was therefore considered a suitable resin for separating the47 kDa and high molecular mass VP2 proteins from the 80 kDa contaminating proteins shown in Figure 2.19. Two major peaks were observed when the sample obtained after TPP containing VP2 was separated on Sephacryl S300 (Figure 2.20B). Reducing SDS-PAGE analysis of the two fractions, followed by staining with Oriole™ Fluorescent Gel Stain (Bio-Rad) that is able to detect proteins at a concentration as low as 0.002 µg, is shown in Figure 2.20C. Lane 1 is the VP2 sample which was loaded onto the MEC column. Fractions from peak one (lanes 2 to 6) contained only high molecular mass proteins and therefore separation of these proteins was successful. Analysis of peak two (lanes 7 to 12) demonstrated unsuccessful separation as VP2 at 47 kDa co-eluted with contaminating proteins. Since it was believed that the high molecular mass proteins in the first peak were VP2 multimers or incorrectly folded VP2, these fractions were pooled for later analysis.

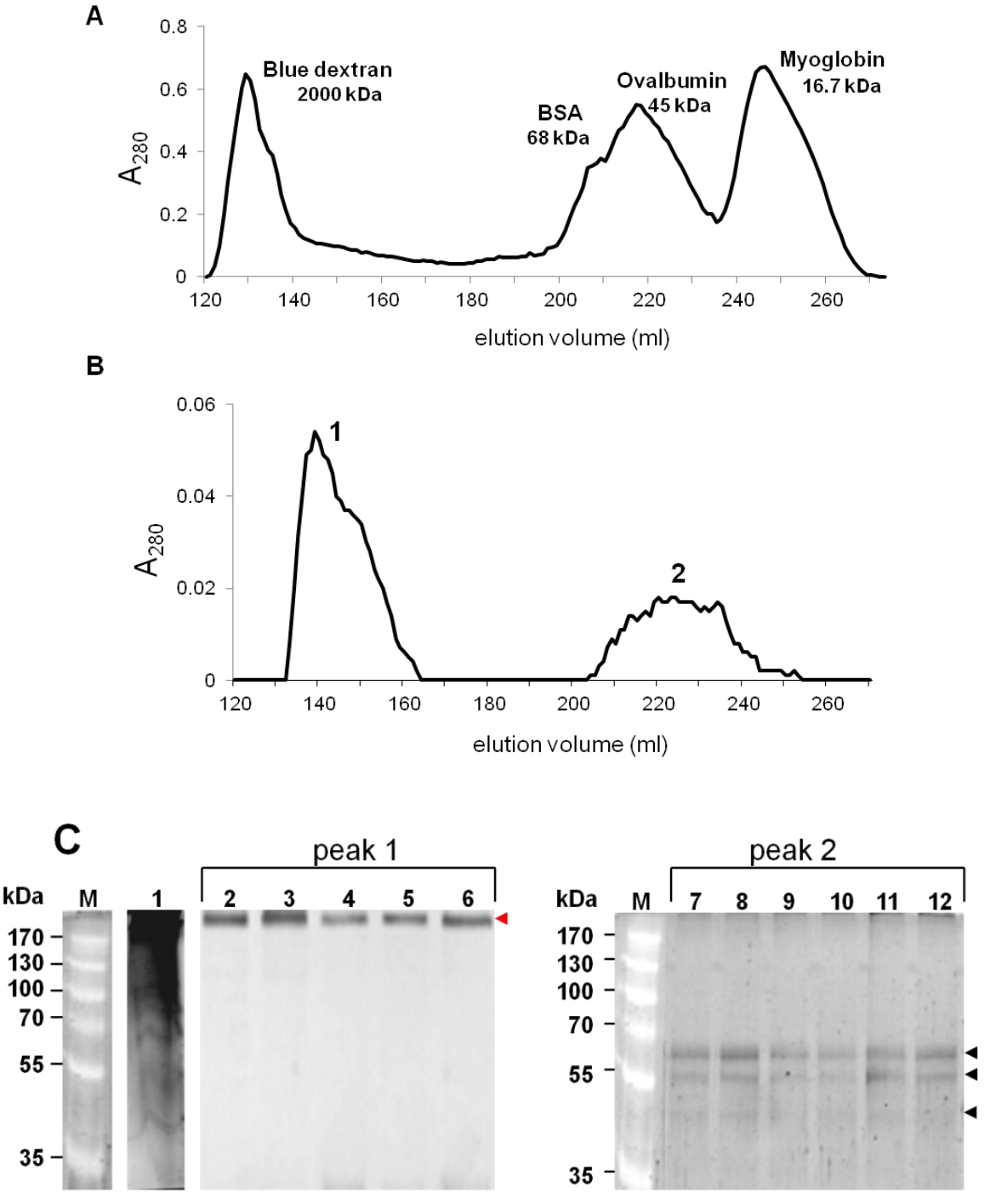

Figure 2.20 Analysis of P. pastoris expressed VP2 purification on a Sephacryl S300 MEC column. (A) Elution of calibration proteins: blue dextran (2000 kDa), BSA (68 kDa), ovalbumin (45 kDa) and myoglobin (16.7 kDa), were used to calibrate the column (25 x 840 mm, flow rate 25 mL/h, MEC buffer). **(B)** Elution profile of VP2 separation on Sephacryl S-300. **(C)** Analysis by 10% reducing SDS-PAGE of elution peaks stained with Oriole™ Fluorescent Gel Stain**.** Lane M, molecular mass marker; lane 1, VP2 sample before separation on MEC; lanes 2-6, elution volume 135, 140, 145, 150 and 155 from peak 1; lanes 7-12, elution volume 208, 212, 221, 226, 233 and 236 from peak 2. The red arrow indicates the VP2 high molecular mass proteins which were efficiently separated from the contaminating proteins and the 47 kDa VP2 protein (black arrows) which could not be efficiently separated from one another.

2.4.4 VP2 peptide selection

The VP2 peptides were designed for raising chicken anti-peptide antibodies that would be used to identify possible IBDV receptor(s). The VP2 amino acid sequence (Met^{1}-Ala^{441}) was analysed with the Predict7™ program (Cármenes *et al.*, 1989) to determine ideal peptide sequences according to hydrophilicity, surface probability, flexibility and antigenicity parameters (Figure 2.21). From the plot three peaks were considered good candidates which are around residues 31, 201 and 401, which all display high hydrophilicity. Antigenicity is relatively low throughout the sequence because this parameter is based on information on the antigenicity of a very small number of proteins (Cármenes *et al.*, 1989). For the production of chicken anti-VP2 peptide antibodies two peptide sequences with high hydrophilicity and surface probability were selected: residues 26 to 39 and 192 to 202 (Table 2.2 and Figure 2.22). In addition to the Predict7™ software, the Cn3D™ program was used to determine the location of the two peptides on the VP2 crystal structure (Figure 2.22B and D). Both peptides were shown to be located in a random loop on the outer surface of the VP2 protein.

Of the two peptides selected for antibody production, the first peptide sequence, VP2-1, consists of 14 amino acid residues which span amino acid residues 26-39 (Figure 2.22A). Peptide VP2-1 demonstrated high hydrophilicity and surface probability towards the centre of the sequence. Antigenicity is low toward the N-terminus and higher toward the centre and C-terminus. Flexibility is constant throughout the sequence. Since the centre of the peptide sequence was higher for all parameters it was decided that the addition of a cysteine residue to either terminus would be acceptable. Therefore a cysteine residue was added at the C-terminus for carrier conjugation (Table 2.2). The second peptide, VP2-2, consists of 11 amino acids which span amino acid residues 192-202 (Figure 2.22C). The VP2-2 peptide demonstrates low hydrophilicity and surface probability at the N-terminus and high toward the C-terminus. Flexibility and antigenicity were relatively constant throughout the sequence although antigenicity is slightly higher toward the C-terminus. A cysteine residue was therefore added at the N-terminus for carrier conjugation and since VP2-2 contained an internal cysteine residue, it was replaced by α-aminobutyric acid (Table 2.2) to avoid conjugation of the carrier to the centre of the peptide which would prevent exposure of the entire epitope to the immune system for antibody production.

A

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         10        20        30        40        50        60        70        80        90       100       110       120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MTNLQDQTQQIVPFIRSLLMPTTGPASIPDDTLEKHTLRSETSTYNLTVGDTGSGLIVFFPGFPGSIVGAHYTLQSNGNYKFDQMLLTAQNLPASYNYCRLVSRSLTVRSSTLPGGVYAL

         130       140       150       160       170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NGTINAVTFQGSLSELTDVSYNGLMSATANINDKIGNVLVGEGVTVLSLPTSYDLGYVRLGDPIPAIGLDPKMVATCDSSDRPRVYTITAADDYQFSSQYQPGGVTITLFSANIDAITSL

         250       260       270       280       290       300       310       320       330       340       350       360
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SVGGELVFQTSVQGLVLGATIYFIGFDGTTVITRAVAADNGLTAGTDNLMPFNLVIPTNEITQPITSIKLEVVTSKSGGQAGDQMSWSASGSLAVTIHGGNYPGALRPVTLVAYERVATG

         370       380       390       400       410       420       430       440
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
SVVTVAGVSNFELIPNPELAKNLVTEYGRFDPGAMNYTKLILSERDRLGIKTVWPTREYTDFREYFMEVADLNSPLKIAGA
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B

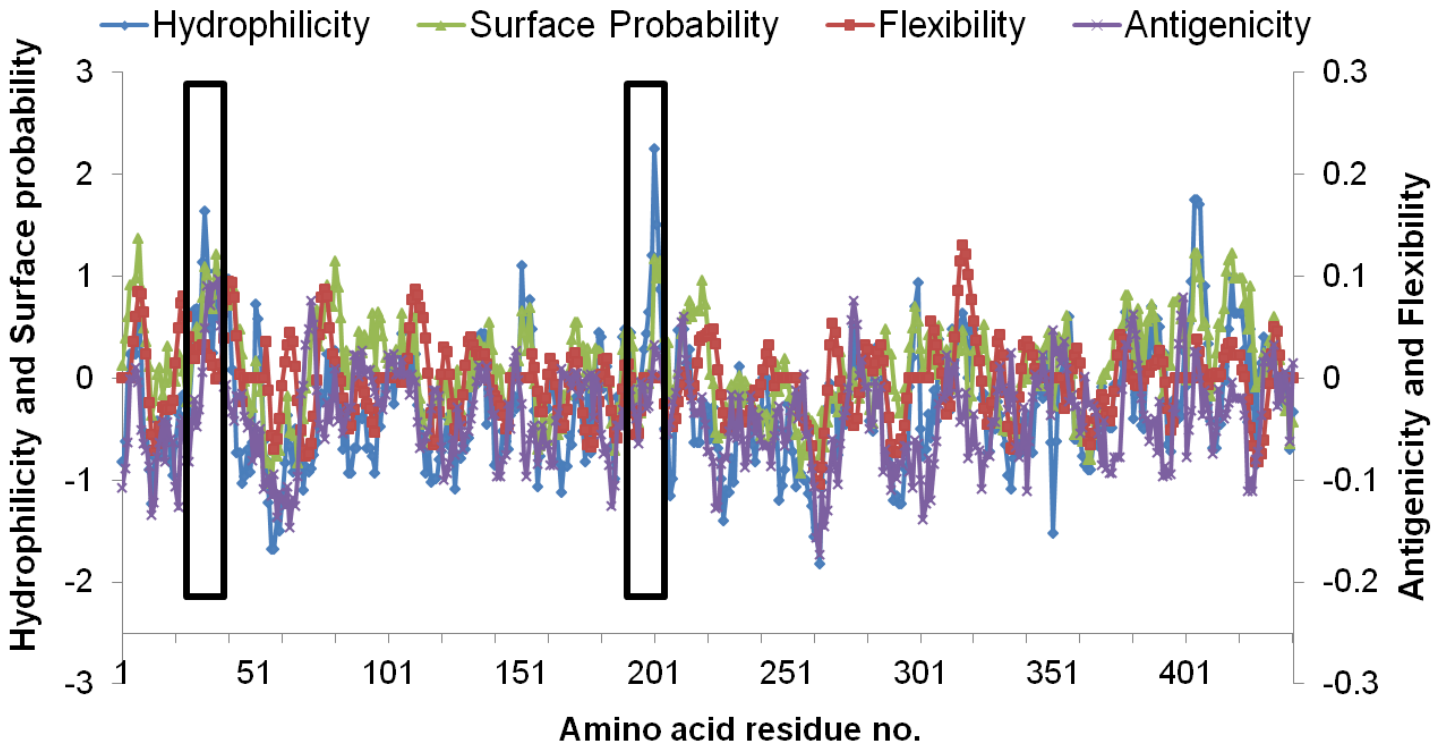

Figure 2.21 Analysis of the VP2 amino acid sequence (residues 1-441) using Predict7™. The amino acid sequence is shown in **(A)** and the Predict7 analysis shown in **(B).** Peptides were selected based on the four paramaters: hydrophilicity, surface probability, flexibility and antigenicity. The regions highlighted by the black rectangles indicate the peptides used for immunisation (for a detailed plot see Figure 2.22).

Table 2.2 Amino acid sequences of the synthetic peptides and their residue positions within the VP2 sequence

Peptide code	Sequence[a]	Residue no.
VP2-1	ASIPDDTLEKHTLR**C**	26-39
VP2-2	**C**KMVAT**Abu**DSSDR[b]	192-202

a Cys in bold was added for carrier conjugation
b α-aminobutyric acid (Abu) was used to replace the internal Cys-residue

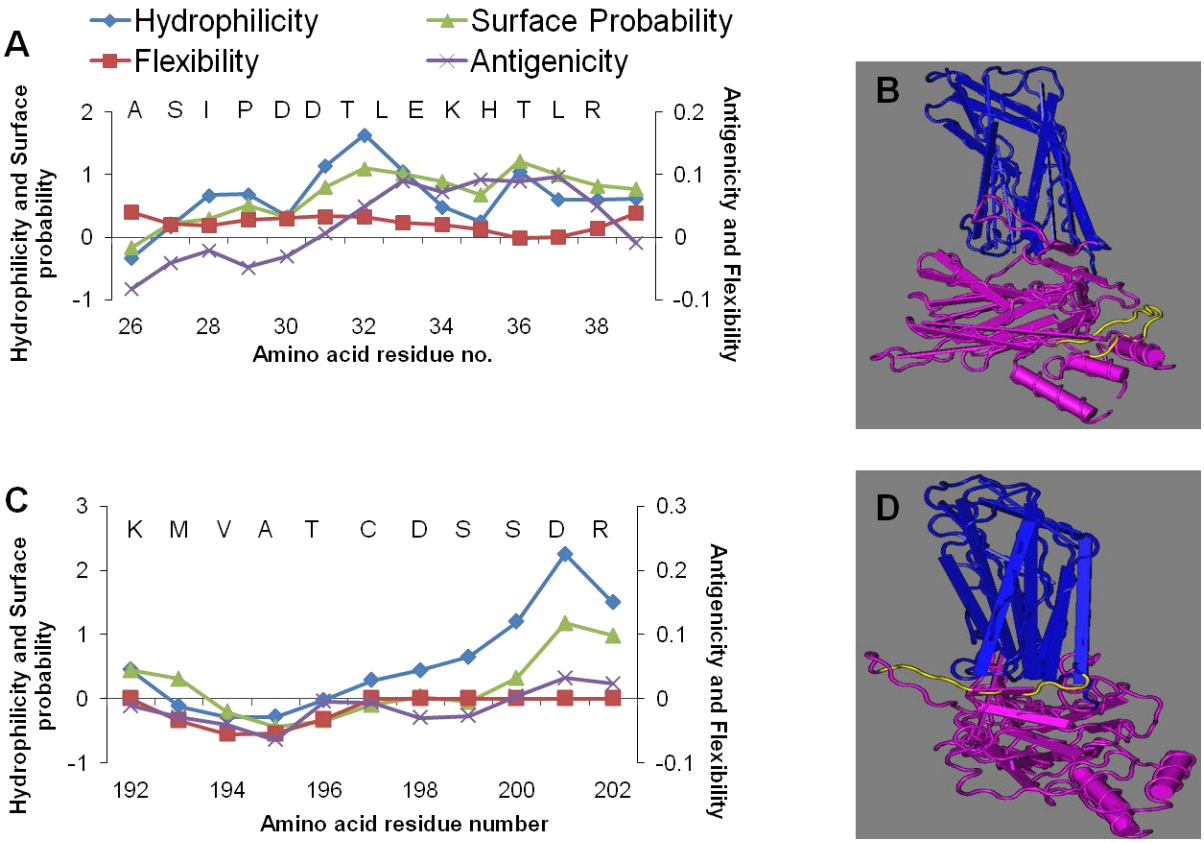

Figure 2.22 Epitope prediction plots of selected VP2 peptides and their respective positions on the VP2 3D structure. (A) VP2-1 amino acid sequence. **(B)** VP2 3D structure demonstrating the VP2-1 peptide sequence. **(C)** VP2-2 amino acid sequence. **(D)** VP2 3D structure demonstrating the VP2-2 peptide sequence. Peptides were selected on the basis of four parameters: hydrophilicity, surface probability, flexibility and antigenicity using Predict7™ software (Cármenes *et al.*, 1989). The VP2 3D structure was viewed using Cn3D™ software to show the localisation of the selected peptides on the structure. The P-domain is displayed in blue and the S and B domains are displayed in pink. The selected peptides are depicted in yellow.

2.4.5 Production and purification of chicken anti-VP2 peptide antibodies

Two pairs of chickens were each immunised with either the VP2-1 or VP2-2 peptide carrier protein conjugate and the immune response monitored over a 13 week period (Figure 2.23). For chicken 1 immunised with VP2-1, an initial peak of antibody production was observed after the first booster immunisation at week 2 followed by a rapid increase in antibodies after the second booster at week 4. Antibody production slowly decreased thereafter and remained relatively constant after the third booster immunisation at week 6 before slowly decreasing again (Figure 2.23A). In chicken 2 immunised with VP2-1, the production of antibodies increased considerably from week 3 up to week 6 and slowly began decreasing in subsequent weeks (Figure 2.23A). Chickens immunised with VP2-2 peptide conjugate showed no immune response which remained constant throughout a 13 week period despite receiving three booster immunisations (Figure 2.23B).

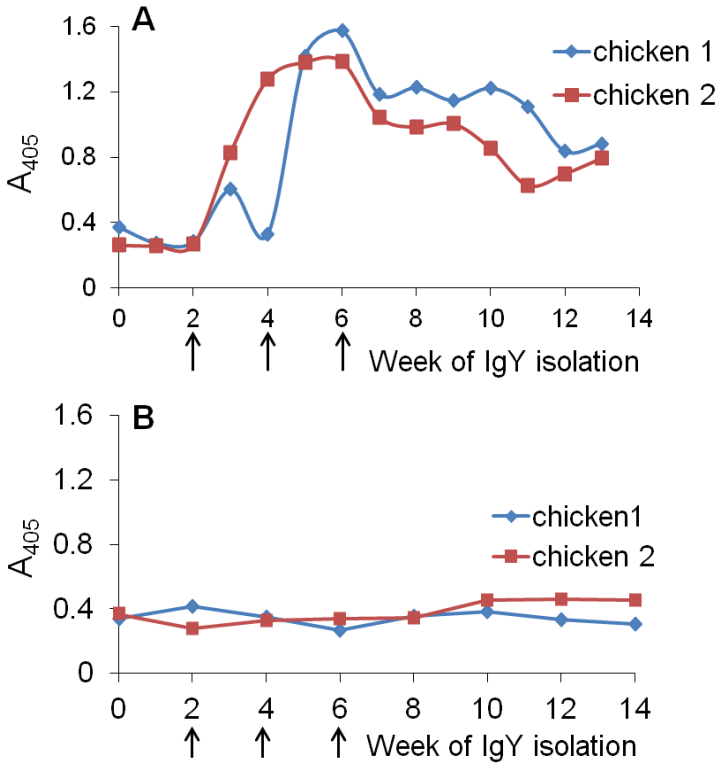

Figure 2.23 ELISA plot demonstrating chicken anti-VP2-1 and chicken anti-VP2-2 peptide antibody production over a 13 week period. Two chickens (chicken 1 and chicken 2) were each immunised with either a VP2-1 or VP2-2 peptide-rabbit albumin conjugate and the antibody production monitored. ELISA plates were coated with 1 µg/mL VP2-1 or VP2-2 peptide and probed with 100 µg chicken anti-VP2-1 or chicken anti-VP2-2 peptide antibodies respectively, followed by detection with rabbit anti-chicken IgY-HRPO conjugate (1:12 000) followed by ABTS/H_2O_2 chromogen-substrate. Arrows indicate weeks at which booster immunisations were administered. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

Titres for antibodies produced over time by chickens immunised with VP2-1 were monitored by ELISA (Figure 2.24A and B). For the first chicken (Figure 2.24A) antibody titres peaked from week 4 and continued to increase up to week 6. Titres decreased slightly in week 7 to week 13. For chicken 2 (Figure 2.24B) the antibody titres increased from week 3 to week 6 and again slowly decreased from week 7 to week 13. Antibodies produced in week 6 demonstrated the best antibody titres while chicken 1 produced higher antibody titres than chicken 2.

Although it was observed that immunising chickens with the VP2-2 peptide-conjugate produced low levels of antibodies, the isolated antibody titres from alternate weeks were in addition analysed on ELISA (Figure 2.25A and B) and the peptide coating concentration increased from 1 µg/mL to 5 µg/mL. Analysis of antibodies produced in chicken 1 (Figure 2.25A) and chicken 2 (Figure 2.25B) demonstrated low antibody titres much like the non-immune control. A small increase was, however, observed from weeks 8 to 11. Antibodies isolated for VP2-2 peptide were therefore not affinity purified due to the relatively low level of chicken anti-VP2-2 peptide antibody production although the non-purified antibodies from weeks 8 to 11 were retained for possible future use.

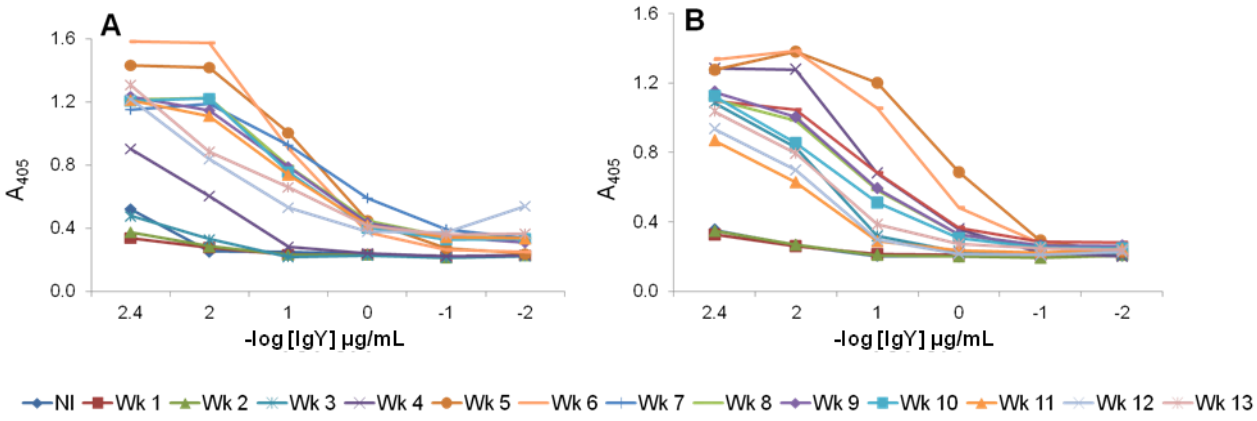

Figure 2.24 ELISA analysis of chicken anti-VP2-1 peptide antibody titres. (A) Anti-VP2-1 peptide antibody titres produced by chicken 1 **(A)** and chicken 2 **(B)** for week 0 to 13 after first immunisation. ELISA plates were coated with 1 µg/mL VP2-1 peptide and probed with a dilution series of antibodies (250 µg/mL – 0.001 µg/mL) isolated from each week before detection with rabbit anti-chicken IgY-HRPO conjugate (1:12 000) followed by ABTS/H_2O_2 chromogen-substrate. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

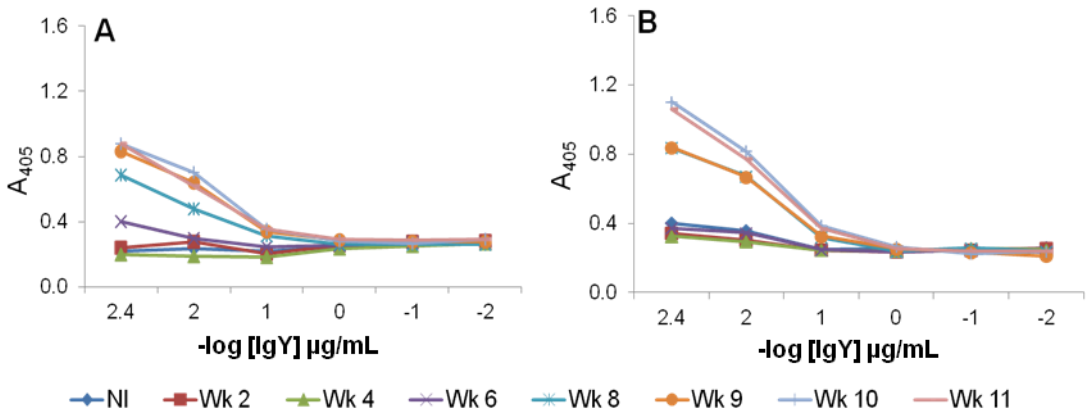

Figure 2.25 ELISA analysis of chicken anti-VP2-2 peptide antibody titres. Chicken anti-VP2-2 peptide antibody titres produced by chicken 1 **(A)** and chicken 2 **(B**) for alternate weeks from week 0 to week 11. ELISA plates were coated with 5 µg/mL VP2-2 peptide and probed with a dilution series of antibodies (250 µg/mL – 0.001 µg/mL) isolated from each week before detection with rabbit anti-chicken IgY-HRPO conjugate (1:12 000) followed by ABTS/H_2O_2 chromogen-substrate. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

The isolated antibodies from the two chickens immunised with the VP2-1 peptide-conjugate were both pooled according to weeks 3-6, weeks 7-11 and 12-13 and affinity purified on a peptide affinity column. The eluted fractions were monitored by measuring absorbance at 280 nm (Figure 2.26). Antibodies from weeks 7-11 were affinity purified in two batches due to the large sample volume and produced the highest concentration of antibodies as indicated by the highest A_{280} readings.

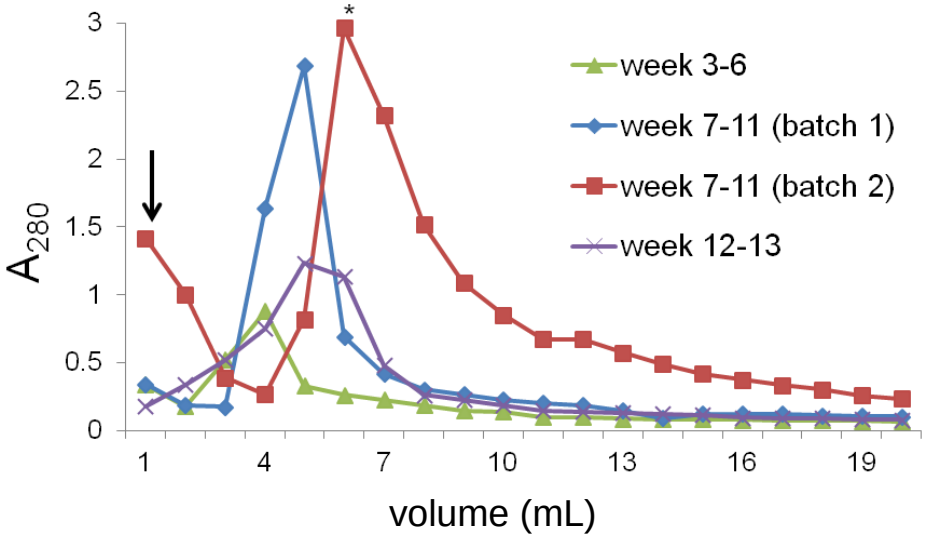

Figure 2.26 Elution profiles of affinity purified chicken anti-VP2-1 peptide antibodies. Pooled, isolated antibodies from weeks 3 to 6, weeks 7 to11 and weeks 12 to 13 were affinity purified.. The arrow indicates the point at which elution began using 0.1 M glycine-HCl and the asterisk (*) indicates points measured at the detection limit of the spectrophotometer.

Recognition of the VP2-1 peptide in an ELISA of affinity purified chicken anti-VP2-1 peptide antibodies was compared to that of antibodies before affinity purification (non-purified) and the unbound fraction (Figure 2.27). The pooled affinity purified antibodies were analysed separately i.e. weeks 3-6 (A), weeks 7-11 (B) and weeks 12-13 (C). Affinity purified antibodies from weeks 7-11 (Figure 2.27B) showed higher recognition of the peptide when compared with the non-purified antibodies and antibodies from the other weeks. All pooled affinity purified antibodies demonstrated higher recognition when compared to unbound, non-purified and non-immune (NI) antibodies although week 12 to 13 (Figure 2.27C) demonstrated a very low level of recognition of the peptide in an ELISA.

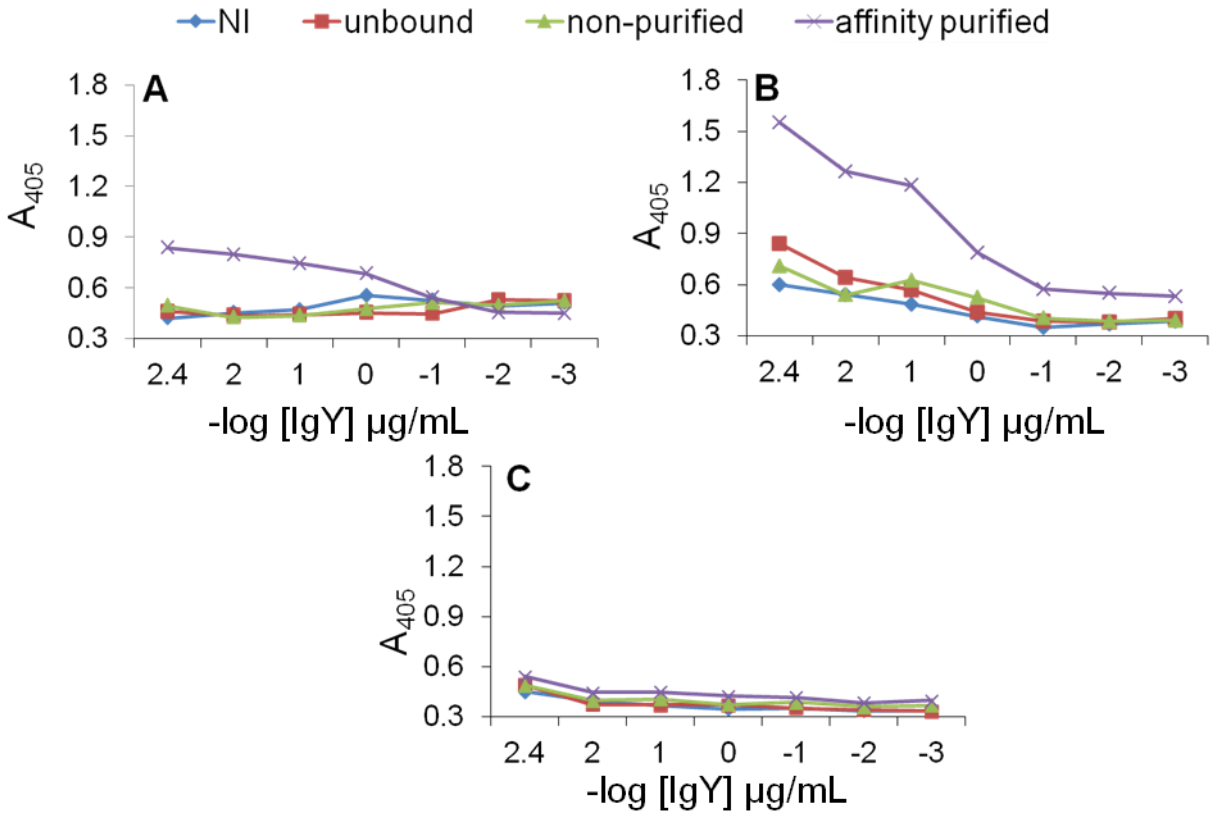

Figure 2.27 Analysis of the recognition of affinity purified chicken anti-VP2-1 peptide antibodies by ELISA. (A) Antibodies purified from weeks 3-6 IgY. **(B)** Antibodies purified from weeks 7-11 IgY. **(C)** Antibodies purified from weeks 12-13 IgY. ELISA plates were coated with 1 µg/mL VP2-1 peptide and probed with a dilution series of antibodies (250 µg/mL - 0.001 µg/mL), followed by detection with rabbit anti-chicken IgY-HRPO conjugate (1:12 000) followed by ABTS/H_2O_2 chromogen-substrate. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

The affinity purified chicken anti-VP2-1 peptide antibodies were analysed to determine whether they could detect recombinant VP2 in an ELISA (Figure 2.28). The high molecular mass proteins expressed in yeast and purified using MEC

(Figure 2.19) which were believed to be VP2 multimers were used in the ELISA. Purified high molecular mass proteins (1 µg/mL) was used to coat ELISA plates and incubated with affinity purified chicken anti-VP2 peptide antibodies. The anti-VP2 peptide antibodies successfully detected the high molecular mass proteins when compared to the non-immune (NI) control further supporting the hypothesis that these proteins were VP2 multimers and was as a result used in subsequent experiments. The weeks 7-11 antibodies showed the best recognition compared to antibodies from the non-immune (NI) control, weeks 3-6 and weeks 12-13 antibodies. The results suggest that the peptides were localised in an accessible position on the protein, as shown by the Cn3D™ - derived structure (Figure 2.22).

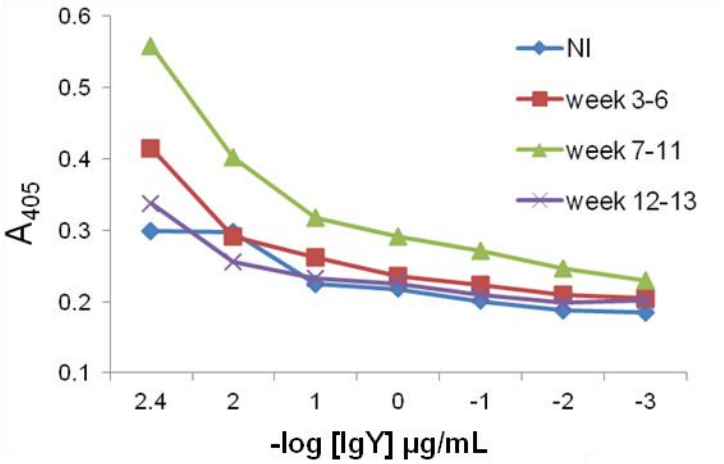

Figure 2.28 Detection of recombinant VP2 by affinity purified chicken anti-VP2-1 peptide antibodies in an ELISA. A coating concentration of 1 µg/mL recombinant VP2 was used and probed with a dilution series of affinity purified chicken anti-VP2 peptide antibodies (250 µg/mL – 0.001 µg/mL) before detection with rabbit anti-chicken IgY-HRPO conjugate (1:12 000) followed by ABTS/H_2O_2 chromogen-substrate. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

2.4.6 Production and purification of chicken anti-VP2 antibodies

Antibodies produced by two chickens immunised with purified VP2 expressed in *E. coli* were pooled according to the weeks of production after the first immunisation and antibody production monitored over a 12 week period (Figure 2.29). Production of antibodies began peaking after the second booster immunisation at week 4 and continued to increase up to week 6. A slight decrease in production was noted after week 6, but remained high and constant up to week 12.

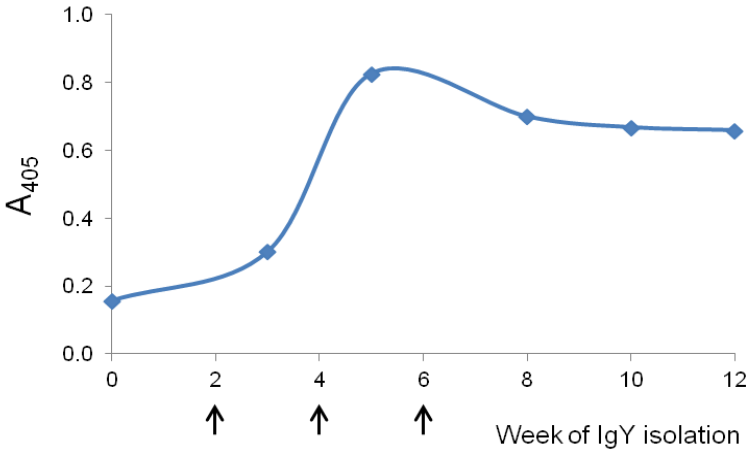

Figure 2.29 ELISA plot demonstrating chicken anti-VP2 antibody production over a 12 week period. Chickens were immunised with purified VP2 expressed in *E. coli* and the antibody production monitored. ELISA plates were coated with 1 µg/mL VP2 and probed with 100 µg chicken anti-VP2 antibodies followed by detection with rabbit anti-chicken IgY-HRPO (1:12 000) and ABTS/H_2O_2 chromogen-substrate. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

The chicken anti-VP2 antibodies isolated from each week after immunisation were further analysed for antibody titre by ELISA (Figure 2.30). Antibodies isolated from every other week from week 3 to week 12 were analysed along with a pre-immune antibody as a negative control. A high antibody titre was observed from week 3 which continued to increase up to week 12 which demonstrated the highest antibody titre.

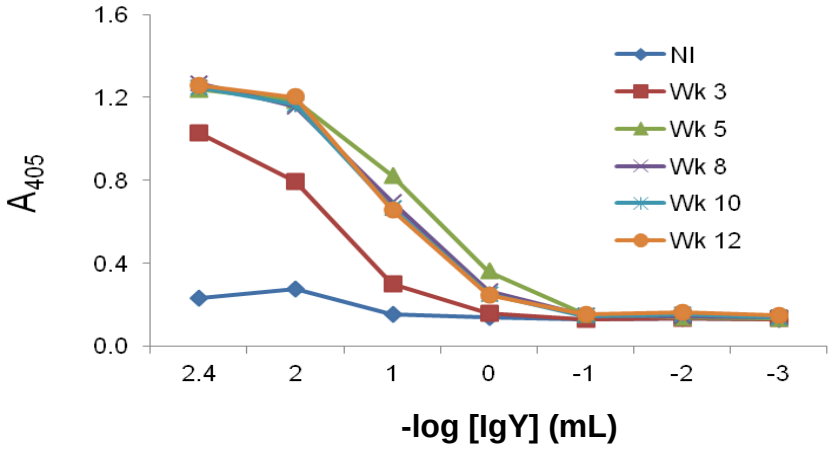

Figure 2.30 Analysis of chicken anti-VP2 antibody titres over a 12 week period by ELISA. Antibodies isolated from weeks 3 to 12 were analysed for levels of titre. ELISA plates were coated with 1 µg/mL recombinant VP2 and probed with a dilution series of antibodies (250 µg/mL – 0.001 µg/mL) from alternate weeks followed by detection with rabbit anti-chicken IgY-HRPO and ABTS/H_2O_2 chromogen-substrate. Each datapoint is the average absorbance reading at 405 nm of duplicate experiments.

Isolated antibodies were pooled into two separate groups, weeks 3 to 7 and weeks 8 to 12, for affinity purification. Each group was affinity purified on a VP2-AminoLink® Plus resin prepared with purified VP2 expressed in yeast. Fractions eluted with a low pH buffer showed that comparable amounts of affinity purified antibody were obtained from the two pools; weeks 3 to 7 and 8 to 12 (Figure 2.31).

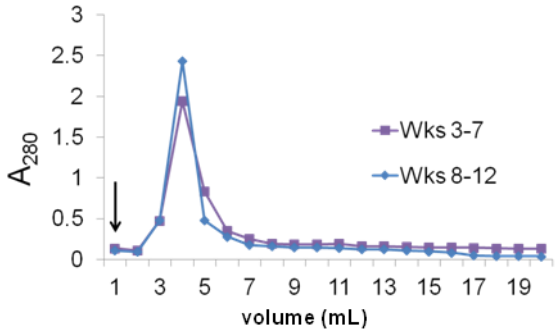

Figure 2.31 Elution profiles of affinity purified chicken anti-VP2 antibodies. Isolated antibodies from weeks 3 to 7 and weeks 8 to 12 were affinity purified. The arrow indicates the point at which elution began.

The affinity purified antibodies were analysed by indirect ELISA and compared with a non-immune (NI) control, unbound antibodies and non-purified antibodies to determine the level of recognition of the VP2 antigen (Figure 2.32). Both affinity purified antibody preparations demonstrated a higher level of recognition compared to that of the non-immune control, non-purified and unbound antibodies (Figure 2.32A and B).The same trend was observed for purified chicken anti-VP2 antibodies of weeks 8 to 12 (Figure 2.32B). Antibodies were therefore successfully affinity purified.

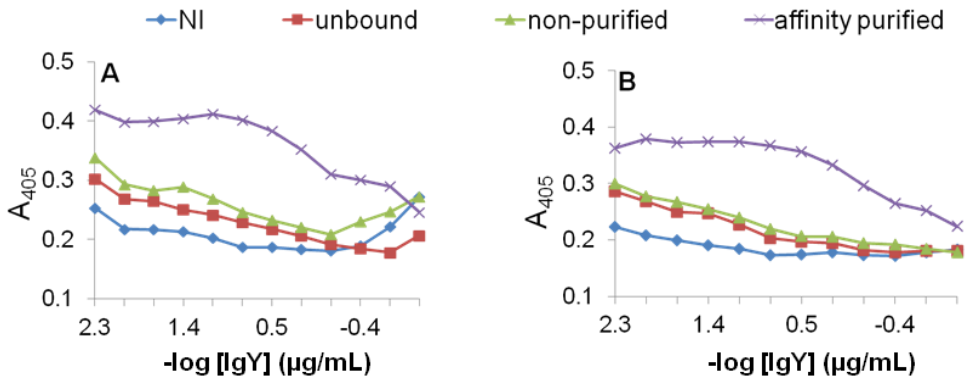

Figure 2.32 Analysis of the recognition of affinity purified chicken anti-VP2 antibodies by ELISA. (A) Antibodies purified from weeks 3-7. **(B)** Antibodies purified from weeks 8-12. ELISA plates were coated with 1 µg/mL recombinant purified VP2 and probed with a dilution series of antibodies (200 µg/mL – 0.1 µg/mL) followed by detection with rabbit anti-chicken IgY-HRPO and ABTS/H_2O_2 chromogen-substrate. Each plot is the average absorbance reading at 405 nm of duplicate experiments.

Since purified VP2 expressed in *P. pastoris* (Figure 2.20C) would be used in a VOPBA to identify possible IBDV receptor(s), chicken anti-VP2 peptide and chicken anti-VP2 antibodies were analysed for their ability to recognise the recombinant protein in a western blot (Figure 2.33). The antibodies demonstrated specificity by not binding to proteins expressed in the non-induced lysate (lane 1). The high molecular mass VP2 proteins were successfully recognised by both chicken anti-VP2 peptide (lane 2) and chicken anti-VP2 (lane 3) antibodies.

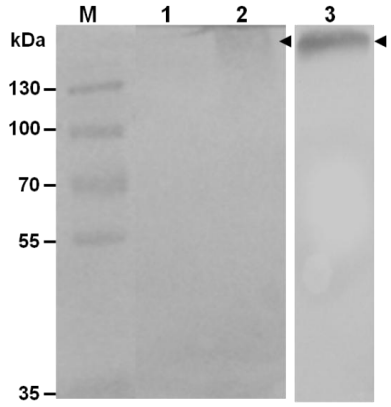

Figure 2.33 Western blot analysis of recombinant VP2 with affinity purified chicken anti-VP2 antibodies and chicken anti-VP2 peptide antibodies. Lane M, PageRuler™ Prestained Protein Ladder; lane 1, non-induced control lysate and purified VP2 expressed in *P. pastoris,* detected with chicken anti-VP2 peptide antibodies (lane 2) and chicken anti-VP2 antibodies (lane 3). Arrow indicates VP2.

2.5 DISCUSSION

Outer capsid protein, VP2, contains neutralising epitopes and is the receptor binding protein of IBDV (Yip *et al.*, 2007). It therefore plays a vital role in IBDV entry. Viral entry is the first step in the virus life cycle and understanding this mechanism could lead to understanding the pathogenesis of the virus as well as allow the successful production of inhibitors or drugs which prevent entry of IBDV into host cells. Most viruses bind to receptors on the host cell membrane to gain entry. The receptors for IBDV entry have not been conclusively identified therefore the objective of the present study was to use potential receptor binding proteins to help identify the receptor(s) of IBDV. The work described in this chapter reports on the recombinant expression of VP2 in bacteria and yeast, purification of recombinant VP2 and production of antibodies against a VP2 peptide and against VP2.

The VP2 coding region was previously cloned into a T-vector and sub-cloned into the pGEX-4T-1 and pET-32a expression vectors. In the current study the VP2 gene

was also sub-cloned into the pPIC9 yeast expression vector and all three expression systems used for the expression of VP2. Proteins expressed using the pGEX-4T-1 system are expressed with an N-terminal GST tag of 26 kDa thus VP2 (47 kDa) would be expressed as a 73 kDa fusion protein. However, expression of VP2 using the pGEX-4T-1 system failed despite increasing the final IPTG concentration used for induction of expression. It is not clear why expression failed in this system as it was previously used to successfully express the IBDV VP4 protease (Vukea, 2011). Taking into account that exact conditions for expression vary for each fusion protein, further optimisation of culture conditions (cell strain, medium composition, temperature and IPTG concentration) failed to improve expression. It is interesting that even though the system expressed VP4, it was unable to express VP2. The VP4 protease is similar to *E. coli* Lon proteases (Birghan *et al.*, 2000, Botos *et al.*, 2004) therefore a reason for non-expression of VP2 in the pGEX-4T-1 system could be codon usage bias much like that experienced in expression of *Plasmodium* proteins in *E. coli* (Baca and Hol, 2000). Since expression in *E. coli* failed using the pGEX-4T-1 vector, expression of VP2 was attempted with the pET-32a vector.

Proteins expressed using the pET-32a vector have an N-terminal 17 kDa thioredoxin and histidine tag (Trx-His tag) thus would express VP2 as a fusion protein of 64 kDa. The pET-32a vector differs from pGEX-4T-1 in that it has a T7 promoter. The *E. coli* BL21(DE3) cells transcribe T7 RNA polymerase therefore auto-induced expression is possible in this system (Jia *et al.*, 2011). Both auto-induced and IPTG induced expression of VP2 were performed. The VP2 protein was successfully expressed as a 64 kDa fusion protein through auto-induction in Terrific broth as inclusion bodies at both 30°C and 37°C. Studies have shown that decreasing the expression temperature (Schein and Noteborn, 1988), co-expression with a chaperone (Goloubinoff *et al.*, 1989) and fusion with a short peptide (Makrides, 1996) could increase the solubility of a recombinant protein. Expression of VP2 with a Trx-His fusion tag and at lower temperatures did not increase the solubility of the recombinantly expressed protein. Previous studies have confirmed that the formation of inclusion bodies is not dependant on the characteristics of the expressed protein such as molecular mass, use of fusion tags or the hydrophobicity of the recombinant protein, but on charge average (Kane and Hartley, 1988, Wilkinson and Harrison, 1991). Additionally, over-expression of proteins can cause the formation of inclusion bodies (Gribskov and Burgess, 1983). Expression of VP2

as inclusion bodies posed a problem, but due to the presence of a His-tag, purification could still be performed under denaturing conditions using a nickel affinity column (QIAGEN instruction manual, 1997). Inclusion bodies were successfully solubilised in 8 M urea. Initially VP2 did not bind efficiently to the resin and some proteins were released during the washing step. In addition, VP2 also eluted with contaminating bacterial proteins. Therefore the protein fractions from the washing step were applied to a cation-exchange chromatography column using step-wise elution with NaCl for further purification and pure VP2 was eluted using 0.5 M NaCl.

In order to increase VP2 yields in addition to purified VP2 obtained using cation-exchange chromatography, the study also took advantage of on-column refolding of the protein. A decreasing gradient wash from 8 to 0 M urea was applied to the nickel column allowing the bound protein to slowly refold. There are a few methods available to refold proteins such as direct dilution which uses refolding buffers to slowly dilute out denaturant, membrane controlled denaturant removal which uses step-wise dialysis and the chromatographic method which uses buffer exchange to remove denaturant (Vallejo and Rinas, 2004). On-column refolding offers several advantages. It is cheap, fast, easily automated and can be applied to a broad range of proteins (Oganesyan *et al.*, 2005). The VP2 protein was successfully purified and renatured by on-column refolding as evidenced by a visible size-shift on SDS-PAGE of the non-reduced refolded protein when compared to the reduced refolded protein.

Additionally, VP2 expression with the pET-32a system in *E. coli* resulted in high molecular mass proteins which are believed to be incorrectly folded VP2. The IBDV VP2 protein has been shown to spontaneously assemble into SVPs during expression (Garriga *et al.*, 2006, Lee *et al.*, 2006b, Dey *et al.*, 2009). The expression systems used in the studies which report on the assembly of SVPs are pESC-URA containing the VP2 coding sequence (Garriga *et al.*, 2006) and the IBDV polyprotein coding sequence (Dey *et al.*, 2009) both expressed in *Saccharomyces cerevisia* and a recombinant baculovirus containing VP2 expressed in Hi-5 cells (Lee *et al.*, 2006b). Assembly of IBDV VP2 has not yet been reported in *E. coli.* Therefore in the present study, electro-elution was used to substantiate the hypothesis that the high molecular mass proteins were in fact VP2. The electro-eluted high molecular mass proteins were observed at the top of the gel as well as at 64 kDa, the expected size of the VP2 fusion protein when analysed by SDS-PAGE which supported the

hypothesis that the high molecular mass proteins were VP2 and explains their recognition by the chicken anti-VP2 antibodies. Superoxide dismutase, an enzyme that catalyses dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide in humans, has been shown to aggregate into high molecular weight, biochemically distinct, insoluble protein complexes much like insoluble inclusion bodies (Johnston *et al.*, 2000). Therefore it is possible that the high molecular mass VP2 observed is in fact incorrectly folded VP2 which aggregates as high molecular mass proteins. Therefore VP2 was successfully expressed and purified using the pET-32a *E. coli* expression system, yielding sufficient amounts of protein to immunise chickens for the production of polyclonal antibodies. Although VP2 expressed well in the pET-32a system, it was obvious that purification of the protein expressed in inclusion bodies posed a problem. This was evident by the low yield of purified protein. Therefore an alternative expression system was explored in order to obtain greater yields of purified protein which could be used to couple to an affinity matrix for the affinity purification of chicken anti-VP2 antibodies and for the use in a VOPBA (Chapter 3).

Since previous studies on VP2 have used eukaryotic expression systems, the present study also explored expressing VP2 in a yeast expression host, *P. pastoris,* in an effort to obtain a higher yield of purified VP2. The *P. pastoris* expression system allows efficient production of secreted proteins (Wu *et al.*, 2005) and provides the advantage of post-translational modification such as glycosylation, proteolytic processing, folding and disulfide bond formation (Aloulou *et al.*, 2006). The VP2 protein was successfully expressed as a 47 kDa protein as expected and as high molecular mass proteins in the *P. pastoris* system as confirmed by western blotting using chicken anti-VP2 antibodies. The VP2 coding sequence has been previously cloned into the *P. pastoris* system with success using pHILD2 and pHILS1 (Pitcovski *et al.*, 2003), pPICZαA (Wu *et al.*, 2005) and pCR 2.1 (Villegas *et al.*, 2008). In the present study the VP2 coding sequence was cloned into the pPIC9 vector for expression in GS115 cells. Previous studies on the expression of VP2 in yeast did not report the expression of high molecular mass proteins (Pitcovski *et al.*, 2003, Wu *et al.*, 2005, Villegas *et al.*, 2008). However, a western blot using anti-IBDV monoclonal antibody detected a 52 kDa recombinant VP2 and proteins greater in size than 75 kDa situated at the top of the gel in the study conducted by Villegas *et al.* (2008) which could be high molecular mass form of VP2. In addition, the high

molecular mass proteins in the current study were sufficiently detected using anti-VP2 peptide antibodies in an ELISA and therefore confirmed to be VP2.

The VP2 expressed in yeast was initially concentrated and purified by the TPP method developed by Pike and Dennison (1989). Three phase partitioning successfully concentrated the yeast expressed supernatant, but failed to remove the contaminating proteins, thus the concentrated sample was further purified using molecular exclusion chromatography. This method separated the high molecular mass proteins from a lower molecular mass fraction containing the 47 kDa VP2 and other contaminating proteins, but was not successful separating the latter. Since the high molecular mass proteins were confirmed to be VP2, this fraction was used for subsequent experiments. Following successful expression of VP2 in *P. pastoris* and purification to homogeneity, the VP2 was used to prepare an affinity column to purify chicken anti-VP2 antibodies. Chickens were deemed to be the best experimental animal to produce antibodies for a number of reasons. The antibody extraction method is fairly simple and straight forward and does not require bleeding to obtain the antibodies since they are packaged into the eggs (Goldring and Coetzer, 2003). In addition the quantity of antibodies isolated from a single egg yolk is comparable to antibodies isolated from bleeding a rabbit every 2 weeks (Gee *et al.*, 2003). Recombinant VP2 expressed in *P. pastoris* was coupled to Aminolink® coupling resin and used to successfully affinity purify chicken anti-VP2 antibodies as evidenced by improved recognition of VP2 in an ELISA compared to IgY before affinity purification and non-immune antibodies.

Peptides were also selected from the VP2 amino acid sequence, coupled to a carrier protein and used to raise polyclonal anti-peptide antibodies in chickens. The idea was to observe whether anti-peptide antibodies could sufficiently recognise recombinantly expressed VP2 and whether the level of recognition would be greater for chicken anti-VP2 peptide than chicken anti-VP2 antibodies. Therefore two peptides, VP2-1 and VP2-2, which displayed high levels of hydrophilicity according to the Predict7™ epitope prediction programme (Cármenes *et al.*, 1989), were selected from the VP2 amino acid sequence. Even though both peptides were localised on the surface of the VP2 protein, higher antibody titres were observed for the chicken anti-VP2-1 peptide antibodies. Since only a small amount of anti-VP2-2 peptide antibodies were produced they were not affinity purified, but retained for possible future use. The chicken anti-VP2-1 peptide antibodies were successfully

affinity purified as observed by the high recognition of purified antibodies to VP2-1 peptide when compared to non-immune and non-purified antibodies in an indirect ELISA. Additionally, the anti-VP2 peptide antibodies recognised VP2 expressed in *P. pastoris* in a western blot and ELISA. Antibodies were also successfully produced against VP2 which supports the notion that VP2 is highly immunogenic as it is the primary host-protective immunogen and therefore the primary target for vaccine development (Chen *et al.*, 2005). The recombinant VP2 appears to maintain the immunogenic properties of the native protein.

In this study, VP2 was successfully expressed and purified in both *E. coli* and *P. pastoris* expression systems. The VP2 protein expressed as a 64 kDa fusion protein in the *E. coli* pET-32a system and as a 47 kDa in the pPIC9 yeast expression system. High molecular weight proteins were also observed in both expression systems. Expression in *P. pastoris* was favoured over *E. coli*, firstly because VP2 expressed as a soluble protein as opposed to inclusion bodies in *E. coli*. Secondly the yield of purified VP2 was greater in *P. pastoris*. VP2 expressed in *E. coli* was used to raise polyclonal antibodies in chickens while VP2 expressed in *P. pastoris* was used to generate an affinity column for the successful affinity purification of chicken anti-VP2 antibodies. Peptides designed from the VP2 amino acid sequence was used to produce anti-VP2 peptide antibodies in chickens. Purified VP2, chicken anti-VP2 and chicken anti-VP2 peptide antibodies will be used for the identification of possible IBDV receptor proteins as described in Chapter 3.

CHAPTER 3

IDENTIFYING POSSIBLE IBDV RECEPTOR(S) USING VIRUS OVERLAY PROTEIN-BINDING ASSAYS AND AFFINITY CHROMATOGRAPHY

3.1 INTRODUCTION

Infectious bursal disease virus remains one of the most economically important diseases in the poultry industry and although there are vaccines available they are often compromised by the presence of maternal antibodies (Corley and Giambrone, 2002) and the continuous emergence of new virulent IBDV strains (Cao *et al.*, 1998b). There remains a need to explore other strategies to control the disease. One of the strategies that has proven successful is developing novel antiviral agents which target the stages or processes that are crucial to the life cycle of the virus, such as virus entry (Sharma *et al.*, 2000, Yuan *et al.*, 2012). Drugs or inhibitors against viral entry, targeting either the viral receptor binding protein or receptor(s) on the host cells, have been successfully developed and have been shown to prevent viral entry such as Enfuvirtide used for the clinical treatment of HIV (Wilen *et al.*, 2012). Enfuvirtide is a short peptide resembling the carboxy-terminal helical region of gp41, a subunit of the HIV envelope which competitively binds target molecules. Studies of the antiviral agents in patients show a significant improvement in antiretroviral activity (Lalezari *et al.*, 2003, Hardy and Skolnik, 2004). It is therefore important to identify both the receptor binding protein on viral capsids or membranes and the receptor(s) of the host cells. The outer capsid protein VP2, is the receptor binding protein of IBDV (Yip *et al.*, 2007); however, the receptor(s) on the bursal cells of the bursa of Fabricius have not yet been conclusively identified.

There are several biochemical methods used to identify cellular receptors, some of which include the virus overlay protein-binding assay (VOPBA), affinity chromatography and the use of anti-receptor antibodies (Smith and Helenius, 2004). The VOPBA is similar to western blotting in that plasma membranes are separated by SDS-PAGE and transferred to nitrocellulose or PVDF membranes. Electrophoretically transferred proteins are incubated with prepared virus and antivirus antibodies are used to detect any proteins which the virus specifically binds to. The alpha-dystroglycan receptor for Lassa fever virus (Cao *et al.*, 1998a),

angiotensin-converting enzyme 2 receptor for SARS coronavirus (Li *et al.*, 2003) and HSP70 and STAT-2 proteins used by the chikungunya virus to establish infection of mammalian cells (Paingankar and Arankalle, 2014) have been identified using the VOPBA method. In affinity chromatography, the virus or receptor binding protein is coupled to an affinity matrix and used to isolate all binding proteins which may represent the receptor proteins. Encephalomyocarditis virus bound to an affinity matrix was used to successfully isolate candidate receptor(s) on human nucleated cells (Jin *et al.*, 1994) and membrane cofactor protein was identified as the Adenovirus receptor by use of affinity chromatography (Trauger *et al.*, 2004). Anti-receptor antibodies are also often used to immunopurify receptors for example a 110 kDa glycoprotein receptor for mouse hepatitis virus (Williams *et al.*, 1990) and the Human membrane cofactor protein (CD46) used by the measles virus (Naniche *et al.*, 1993) were purified using monoclonal antibodies.

Although the IBDV receptor(s) has not been conclusively identified, several different membrane proteins have been shown to interact with IBDV. The virus has been shown to target surface IgM positive lymphocytes (Hirai and Calnek, 1979) which led researchers to believe that IgM was the IBDV receptor although this was later shown to be incorrect (Ogawa *et al.*, 1998). Nieper and Muller (1996) performed saturation and competition experiments using radiolabelled IBDV of serotype I and II and chicken embryo fibroblast (CEF) cells which are susceptible to both serotypes. The study demonstrated that CEF cells have receptor proteins which are specific to both serotypes as well as specific receptors for each serotype (Nieper and Muller, 1996). Furthermore, VOPBA results showed two proteins of 40 and 46 kDa on the surface of CEF cells which specifically bound to both serotypes of IBDV (Nieper and Muller, 1996). In a study conducted by Ogawa *et al.* (1998), IBDV binding to B-cells was demonstrated, however when treated with proteases and N-glycosylation inhibitors, binding was inhibited. These results suggest that the receptor is a protein which could be N-glycosylated (Ogawa *et al.*, 1998).

Later, VOPBA experiments were performed using plasma membranes of LSCC-BK3 cells from a chicken B lymphoblastoid cell line which is also susceptible to IBDV infection (Setiyono *et al.*, 2001). Three proteins of 70, 82 and 110 kDa were shown to specifically bind to IBDV (Setiyono *et al.*, 2001). Lin *et al.* (2007) were the first study to identify a protein which forms part of the receptor and is essential for IBDV

entry. Instead of IBDV particles, the study used subviral particles (SVPs) formed by VP2 (Coulibaly *et al.*, 2005, Lin *et al.*, 2007). The SVPs were cross-linked to immobilised Ni^{2+} ions on an affinity matrix and used to affinity purify binding proteins of DF-1 cells. Purified protein was identified as chicken heat shock protein 90 (cHsp90) by mass spectrometry (Lin *et al.*, 2007) and VOPBA using IBDV viral particles further confirmed the result. Furthermore the infection of DF-1 cells by IBDV was also shown to be inhibited by cHsp90 and by anti-cHsp90 antibodies.

The objective of this part of the present study was to identify possible IBDV receptor(s) that are present on the bursal membranes using the receptor binding protein VP2. The approach was to detect any bursal membrane proteins which interact with IBDV using a conventional and modified VOPBA and purifying possible IBDV binding proteins using affinity chromatography on a VP2-coupled affinity matrix. Plasma membrane proteins were therefore isolated from the bursae, separated by SDS-PAGE and transferred onto nitrocellulose. For the conventional VOPBA, isolated IBDV was incubated with the nitrocellulose containing transferred plasma membrane proteins. Any proteins which interacted with IBDV were detected using either chicken anti-VP2 or chicken anti-VP2 peptide antibodies. The modified VOPBA incorporated VP2, in place of IBDV, to determine if the interactions would be comparable. The IBDV binding proteins of the plasma membrane were also purified on a VP2-coupled affinity matrix and further analysed by mass spectrometry.

3.2 MATERIALS

The IBDV infected bursae were obtained from Allerton Regional Veterinary laboratory, Pietermaritzburg, South Africa and non-infected bursae from Ukulinga research and training farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Vero cells were available as glycerol stocks. Minimum essential medium (MEM), HEPES and ProteoPrep® membrane extraction kit were purchased from Sigma-Aldrich-Fluka (Steinheim, Germany). Heat inactivated foetal calf serum (FCS) was purchased from Gibco (Paisley, UK). Sterile membrane filters (0.2 µm and 0.45 µm) were purchased from Pall (Ann Arbor, USA) and culture flasks (75 cm^2) were obtained from Corning (New York, USA). PageRuler™ plus prestained protein ladder was purchased from Fermentas (Vilinius, Lithuania).

3.3 METHODS

3.3.1 Preparation of plasma membranes from the bursa of Fabricius

Plasma membranes from the bursae were prepared according to Nieper and Muller (1998). Briefly, fresh bursae were suspended in washing buffer (25 mM HEPES, 154 mM NaCl, 0.5 mM $MgCl_2$, pH 7.4) and excised carefully with scissors. The cells were washed three times in washing buffer before resuspending in three-fold (w/v) excess of ice-cold homogenisation buffer (25 mM HEPES, 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 µM pepstatin A, pH 8.0) and incubated on ice for 10 min. The resuspended cells were homogenised in a Potter S homogeniser by 30-40 strokes and restoration buffer (25 mM HEPES, 600 mM NaCl, 0.5 mM $MgCl_2$, pH 8.0) added immediately to a final concentration of 154 mM NaCl to restore isotonic conditions. EDTA was added to the homogenate to a final concentration of 1 mM and the homogenate centrifuged (5 000 *x g*, 10 min, 4°C). The pellet was discarded and two parts of the supernatant were layered over one part of a 25% (w/v) sucrose cushion made up in homogenisation buffer before ultracentrifugation (100 000 *x g*, 1 h, 4°C). The plasma membranes were collected at the interface which was visible as a white band and diluted in washing buffer before sedimentation by ultracentrifugation (100 000 *x g*, 30 min, 4°C). The white band at the interface was collected and resuspended in washing buffer. Before SDS-PAGE analysis the resuspended isolate was concentrated by adding 10 µL of 5% (w/v) SDS and 10 µL 3 M KCl to 100 µL resuspended isolate in a microfuge tube and mixed together by inverting. The mixture was centrifuged (12 000 x *g,* 2 min, RT), the supernatant discarded and the precipitate resuspended in 10 µL of gel buffer (0.5 M Tris-HCl, pH 6.8) and 10 µL of reducing treatment buffer [0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8].

Plasma membranes were also prepared using the ProteoPrep® membrane extraction kit (Sigma-Aldrich-Fluka, Steinheim, Germany) according to the manufacturer's instructions with minor modifications. Briefly, excised fresh bursae (1 g) was suspended in ice cold Soluble cytoplasmic and loosely-bound membrane protein extraction reagent (8 mL) (Sigma-Aldrich-Fluka, Germany) before disruption by homogenisation using a Potter S homogeniser by 30-40 strokes. Ice cold Soluble cytoplasmic and loosely-bound membrane protein extraction reagent (50 mL) was added to the homogenate and slowly stirred on ice for 1 h before being subjected to

ultracentrifugation (115 000 *x g,* 1 h, 4°C) to pellet membranes and membrane proteins. The supernatant was discarded and the membrane pellet was thoroughly resuspended and washed twice in 2 mL milliQ water with centrifugation between washes (20 000 *x g,* 20 min, 4°C). Thereafter the pellet was resuspended in Protein extraction reagent type 4 (2 mL) (Sigma-Aldrich-Fluka, Germany) and sonicated on ice (4 x 15 s pulses) before centrifugation (14 000 *x g,* 45 min, 15 °C) to pellet cell debris. The supernatant containing plasma membranes was retained and the concentration determined, before analysis by SDS-PAGE (Section 2.3.5.2).

3.3.2 IBDV isolation and cell culture

3.3.2.1Isolation of IBDV from infected bursae samples

The IBDV virus particles were isolated from infected bursae according to Fernandez-Arias *et al.* (1998) with some modifications. Briefly, homogenisation buffer (0.02 M Tris-HCl, pH 7.8) was added in an equal volume to excised infected bursae and homogenised using a Potter S homogeniser. The cell debris was removed with centrifugation (17 000 *x g*, 15 min, 4°C) and one part supernatant was layered on two parts of 40% (w/v) sucrose in homogenisation buffer and ultracentrifuged (86 000 *x g*, 2.5 h, 4°C). The supernatant was discarded and the pellet was thoroughly resuspended in homogenisation buffer. The resuspended pellet was layered over half its volume of 1.37 g/cm^3 CsCl solution and ultracentrifuged (86 000 *x g*, 6 h, 4°C). The virus band which was observed towards the top of the gradient was removed by aspiration using a Pasteur pipette and resuspended in homogenisation buffer before dialysing in buffer (0.01 M Tris-HCl buffer, pH 7.8) overnight to remove CsCl. The dialysed sample was layered again over half its volume of a 1.27 g/cm^3 CsCl solution and ultracentrifuged (86 000 *x g*, 6 h, 4°C). The band containing virus was collected and resuspended in homogenisation buffer and analysed by SDS-PAGE (Section 2.3.5.2).

3.3.2.2Preparation of Vero cell monolayers

The preparation of Vero cell monolayers were carried out according to Hussain and Rasool (2005) with a few modifications. A vial of cryogenic Vero cells (2 mL) at its twentieth passage were removed from liquid nitrogen and thawed. The cells were transferred into a 15 mL tube and the volume made up to 6 mL with complete MEM [MEM base powder, 26 mM $NaHCO_3$, 10% (w/v) FCS, 1% (w/v) penicillin-

streptavidin]. The Vero cell suspension was split (3 x 2mL) and transferred to culture flasks (75 cm^2). To each flask complete MEM (10 mL) was added and incubated at 37°C with 5% CO_2. The flasks were examined twice daily under an inverted microscope (Olympus CK40, Japan) for the formation of a 100% confluent monolayer (48 h). The 100% confluent monolayer was subcultured by removing spent medium and washing twice with pre-warmed PBS (3 mL). Cells were trypsinised and brought into suspension using pre-warmed 0.25% trysin-EDTA (3 mL) and incubated for 30 min at 37°C with 5% CO_2. Cells were lifted by gently hitting the side of the flasks. Complete MEM (6 mL) was added to each flask before splitting cells three way and transferring to new culture flasks (75 cm^2). The volume was made up to 6 mL with complete MEM before incubation at 37°C with 5% CO_2. The cells were examined twice daily under an inverted microscope for the formation of a 90% confluent monolayer.

3.3.2.3Infection of Vero cell monolayers with IBDV and harvesting of IBDV

Infection of Vero cell monolayers was carried out according to Ahasan *et al.* (2002) with some modifications. Isolated IBDV virus (Section 3.3.2.1) was used to infect the 90% confluent monolayers. Briefly, the spent medium was removed and cell monolayers were washed twice with prewarmed PBS (3 mL). To each flask 1 mL IBDV inoculum was added by using a 0.2 µm pore filter steriliser. The inoculum was spread evenly over the Vero cell monolayer and incubated at 37°C for 1 h with intermittent rotation to ensure absorption. Two culture flasks were kept as non-induced controls. After allowing virus absorption, complete MEM (5 mL) was added to each culture flask and incubated at 37°C with 5% CO_2. Flasks were examined twice daily under an inverted microscope for cytopathic effects (96 h). The virus was harvested by three freeze-thaw cycles according to Peilin *et al.* (1997). Medium containing virus was transferred to 10 mL tubes, frozen overnight at -20°C and thawed at RT. This freeze-thaw process was repeated three times before the virus suspension was centrifuged (4 420 x *g*, 5 min, 4°C) to pellet cell debris and the supernatant was stored at -80°C till further use.

3.3.3 VOPBA to identify possible receptor proteins to which IBDV binds

The VOPBA was carried out according to Karger and Mettenleiter (1996) with some modifications. Briefly, isolated plasma membrane proteins (25 µg) were separated

on a 10% reducing SDS-PAGE (Section 2.3.5.2) and transferred electrophoretically to nitrocellulose at 30 V in blotting buffer [20 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, 1% (w/v) SDS] overnight. The nonspecific sites on the membrane were blocked with 5% (w/v) non-fat milk in TBS (20 mM Tris, 200 mM NaCI, pH 7.4) for 1 h. Thereafter the membrane was washed with TBS (3 x 5 min) and incubated for 2 h with either purified IBDV (1:50, according to a previous study conducted in the laboratory) or purified VP2 (50 µg) made up in 0.5% (w/v) BSA-TBS. Following incubation, the membrane was washed in TBS (3 x 5 min) and incubated with either chicken anti-VP2 or chicken anti-VP2 peptide antibodies (100 µg) made up in 0.5% (w/v) BSA-TBS for 2 h. The membrane was washed in TBS (3 x 5 min) and incubated in rabbit anti-chicken IgY secondary antibody conjugated to horse radish peroxidase (HRPO) for 1 h. The membrane was washed with TBS (3 x 5 min) before immersing in chromogen/substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, 0.0015% (v/v) H_2O_2 in TBS), allowed to develop in the dark (3-5 min) until bands were clearly evident against a lightly stained background and thereafter transferred to distilled water.

3.3.4 Affinity purification of IBDV receptor proteins

A VP2 coupled AminoLink® column (1 mL) (Section 2.3.4.5) was used to affinity purify possible IBDV receptor proteins. A 1 M NaOH solution was used to clean and regenerate the column before equilibrating with 20 column volumes of washing buffer [100 mM sodium phosphate buffer, pH 6.5, 0.02% (w/v) NaN_3] at a flow rate of 10 mL/h. Isolated plasma membranes (Section 3.3.1) were thawed and filter sterilised with a 0.45 µm pore size filter before circulating overnight on the affinity column at 4°C. The unbound fraction was collected and the affinity column was washed with 10 column volumes of washing buffer before applying elution buffer (100 mM glycine-HCl buffer, pH 2.8). Fractions were collected (0.5 mL) and A_{280} readings measured. Fractions containing protein were pooled and analysed by 10% reducing SDS-PAGE and stained with Coomassie blue R-250 (Section 2.3.5.2).

3.3.5 Tandem Mass Spectrometry

Affinity purified proteins were prepared for mass spectrometry by separating the sample by SDS-PAGE and staining with Coomassie blue R-250 (Section 2.3.5.2). The protein bands were excised and MS analysis conducted (CSIR BioSciences,

Pretoria, South Africa). Protein bands were in-gel trypsin digested according to Shevchenko *et al.* (2007). Briefly, protein bands were excised from the gel and destained in 100 mM NH_4HCO_3/acetonitrile (1:1 v/v) for 30 min followed by incubation at RT with acetonitrile until gel pieces became white. Protein digestion was carried out in a negligible volume (~50 µL, depending on size of gel piece) of 10 ng/µL trypsin overnight at 37°C. The digested sample was resuspended in 35 µL 2% acetonitrile/0.2% formic acid before analysis on a Dionex Ultimate 3000 ELITE mass spectrometer (Thermo Scientific, Roskilde, Denmark). The ProteinPilot™ v4.0.8085 software which uses the Paragon™ Algorithm was used for comparison of the obtained MS/MS spectra with protein sequences in a Uniswiss 2011 database. Proteins with threshold above ≥ 99.9% confidence were reported.

3.4 RESULTS

3.4.1 Analysis of plasma membrane isolation

Plasma membrane proteins were isolated from the bursa of Fabricius of healthy chickens using two methods. The first was a method described by Nieper and Muller (1998) and the second, a ProteoPrep® membrane extraction kit based on protocols described by Molloy *et al.* (1998) and Herbert *et al.* (1998). Reducing SDS-PAGE was used to analyse the isolated plasma membranes (Figure 3.1). A low yield was obtained with the conventional method described by Nieper and Muller (1998), although faint protein bands of about 40, 43, 60 and 90 kDa were observed after concentrating the plasma membrane isolate (Figure 3.1, lane 1). A greater yield was obtained using the ProteoPrep® membrane extraction kit and further concentrating steps were not required before analysis by SDS-PAGE. Prominent protein bands of approximately 40, 43, 70, 90 kDa and a high molecular weight protein were observed (Figure 3.1, lane 2). Three prominent protein bands of 40, 43 kDa and a high molecular weight protein were observed by Nieper and Muller (1998) which correlates well with results obtained using the extraction kit.

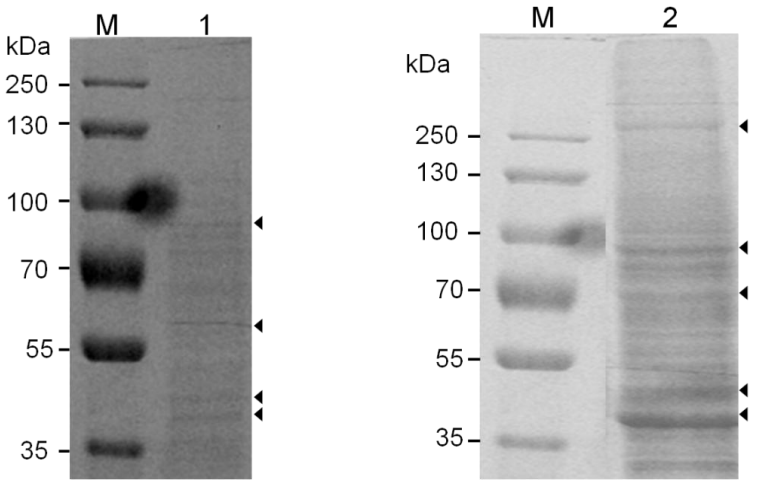

Figure 3.1 Analysis of isolated plasma membrane proteins separated by 10% reducing SDS-PAGE stained with Coomassie Blue R-250. Lane M, PageRuler plus prestained protein ladder; lane 1, membranes isolated using a conventional method and lane 2 membranes isolated using an extraction kit. Arrows indicate major proteins.

3.4.2 The isolation of IBDV from infected bursae and the infection of Vero cells

Infectious bursal disease virus was isolated from IBDV infected bursae and analysed by reducing SDS-PAGE (Figure 3.2). The virus comprises two structural proteins, VP2 (47 kDa) and VP3 (32 kDa), which together form the capsid and make up 80-90% of the total protein of birnaviruses (Dobos, 1996). Three non-structural proteins of IBDV include VP1 (90 kDa), VP4 (28 kDa) and VP5 (17 kDa). The structural proteins, VP2 and VP3 were observed at an expected size of 47 and 32 kDa, respectively (lane 1). A protein band at 28 kDa (likely to be VP4) was also observed.

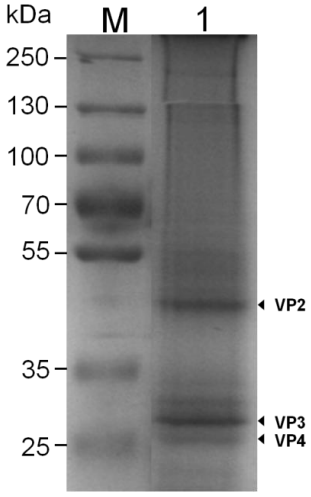

Figure 3.2 Analysis of isolated IBDV by 10% reducing SDS-PAGE stained with Coomassie Blue R-250. Lane M, PageRuler Plus Prestained Protein Ladder; lane 1, isolated IBDV. Arrows indicate protein bands that correspond to structural proteins VP2 and VP3 and the VP4 protease.

Isolated IBDV was propagated in Vero cells (Figure 3.3). The morphology of the Vero cells before infection can be described as fibroblast-like (Figure 3.3A). Following infection, cytopathic effects were observed 96 h post-infection (Figure 3.3B). The cells displayed a change from a fibroblast-like morphology to a more rounded morphology with visible aggregates.

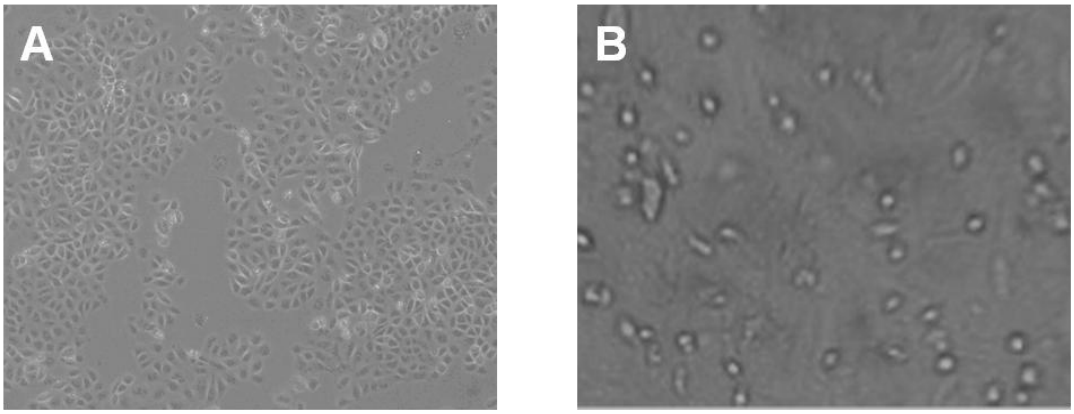

Figure 3.3 Photo images of Vero cells taken using an inverted microscope. (A) A 90% confluent monolayer of Vero cells grown in complete MEM growth medium. **(B)** Cytopathic effects of IBDV on Vero cell monolayer 96 h post-infection (magnification 100x).

3.4.3 Identifying possible IBDV receptor proteins

3.4.3.1 IBDV binding proteins detected using VOPBA experiments

A VOPBA was used to identify possible receptor(s) or IBDV binding proteins on the plasma membranes isolated from the bursae using either chicken anti-VP2 or chicken anti-VP2 peptide antibodies (Figure 3.4). Before the VOPBA was performed, VP2, IBDV, chicken anti-VP2 and chicken anti-VP2 peptide antibody concentrations were optimised by methodically varying one parameter at a time. In the VOPBA experiments, VP2 bound to two proteins of 70 and 32 kDa (Figure 3.4A, lane 2) which was recognised by chicken anti-VP2 antibodies. No detection was observed using chicken anti-VP2 peptide antibodies (Figure 3.4B, lane 2). A 32 kDa protein was observed binding to IBDV as recognised by chicken anti-VP2 antibodies (Figure 3.4C, lane 2) while no detection was observed using chicken anti-VP2 peptide antibodies (Figure 3.4D, lane 2). It is expected that the use of chicken anti-VP2 antibodies would identify more proteins than the chicken anti-VP2 peptide antibodies as the anti-peptide antibodies are targeted to a specific region on the

protein in comparison to anti-protein antibodies which recognise many epitopes on the protein.

The IBDV particles cultured in Vero cells (Figure 3.4, lane 3) and recombinant VP2 (Figure 3.4, lane 5) were also separated by SDS-PAGE to serve as positive controls for the binding of the primary antibodies. Both chicken anti-VP2 and chicken anti-VP2 peptide antibodies recognised the recombinant VP2 at its expected size of 45 kDa and the high molecular mass proteins (Figure 3.4, lane 5), but was unable to detect native VP2 in the isolated virus (Figure 3.4, lane 3). Since IBDV was able to infect Vero cells, the cells were also separated by SDS-PAGE to determine if they would yield comparable results to the bursal membrane. Neither chicken anti-VP2 nor chicken anti-VP2 peptide antibodies detected the proteins on Vero cells suggesting that neither VP2 nor IBDV bound to any proteins on Vero cells (Figure 3.4, lane 4).

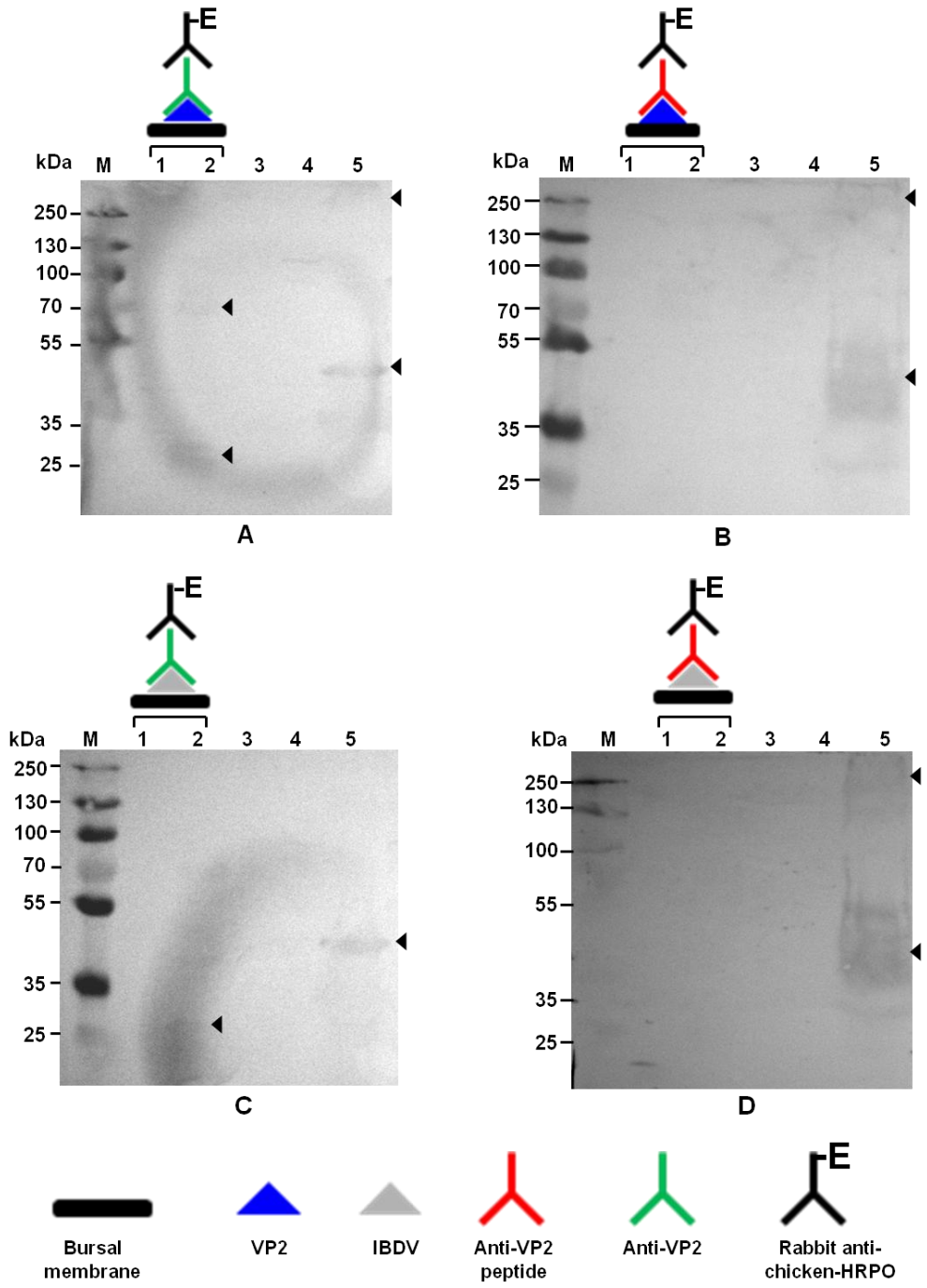

Figure 3.4 VOPBA analysis of possible IBDV receptor proteins. (A) VOPBA probed with 50 µg/mL VP2, 100 µg/mL chicken anti-VP2 antibodies and 1:12000 rabbit anti-chicken antibodies. **(B)** VOPBA probed with 50 µg/mL VP2, 100 µg/mL chicken anti-VP2 peptide antibodies and 1:12000 rabbit anti-chicken antibodies. **(C)** VOPBA probed with 1:50 IBDV, 100 µg/mL chicken anti-VP2 antibodies and 1:12000 rabbit anti-chicken antibodies. **(D)** VOPBA probed with 1:50 IBDV, 100 µg/mL chicken anti-VP2 peptide antibodies and 1:12000 rabbit anti-chicken antibodies. Lane M, PageRuler plus prestained protein ladder; lanes 1 and 2, isolated plasma membrane proteins; lane 3, isolated IBDV; lane 4, Vero cells and lane 5, VP2. Arrows indicate proteins which were detected.

3.4.3.2Affinity purification of possible IBDV receptor proteins

Affinity chromatography was used for the isolation of possible IBDV receptor(s) from bursae and the proteins were analysed by reducing SDS-PAGE (Figure 3.5). A VP2 coupled AminoLink® column was used to isolate the receptor(s) by passing isolated plasma membranes of the bursae (lane 1) through the column. During the affinity purification, non-specific proteins were collected in the unbound fraction (lane 2) and the column was washed to remove any additional non-specific binding proteins (lane 3). Finally, all proteins which strongly bound to VP2 were eluted using a low pH buffer (lanes 4 and 5). Four prominent VP2 binding proteins were observed with molecular masses of approximately 70, 60, 45 and 32 kDa. These protein molecular masses correlate with the sizes that have been reported in previous studies. A VOPBA conducted by Setiyono *et al.* (2001) and Nieper and Muller (1996) reported on IBDV specifically binding to proteins of 70 kDa and 46 kDa respectively, while a study previously conducted in our laboratory reported on a 32 kDa protein as a predominant protein observed binding to IBDV (Edwards, 2000). Reports could not be established which identify IBDV interacting with a 60 kDa protein.

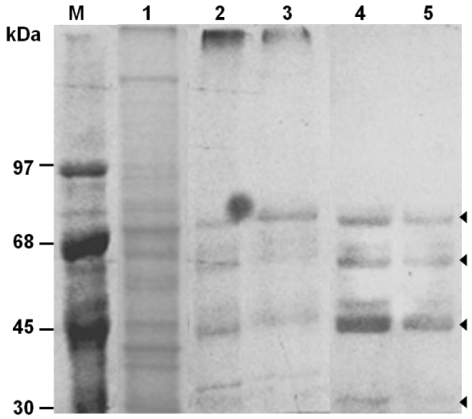

Figure 3.5 Analysis of possible IBDV receptor proteins eluted from a VP2 affinity column. Proteins were separated by 10% reducing SDS-PAGE and stained with Coomassie Blue R-250. Lane M, molecular weight marker; lane 1, isolated plasma membrane proteins, lane 2, unbound fraction; lane 3, wash fraction and lanes 4 and 5, elution fractions. Arrows indicate the four eluted proteins which were further analysed.

3.4.3.3Mass spectrometry of affinity purified proteins

Tandem mass spectrometry was performed to identify the affinity purified proteins. Mass spectrometry analysis identified one protein with common peptides to the 70 kDa protein, two proteins with common peptides to the 60 kDa protein, two proteins

with common peptides to the 45 kDa protein and three proteins with common peptides to the 32 kDa protein (Table 3.1). All peptides were identified with at least 95% confidence and the proteins identified demonstrated identity of more than two peptides to the affinity purified proteins. Bacterial contamination was ruled out. Peptides of the immunoglobulin gamma chain showed identity to peptides of all four of the affinity purified proteins which suggests the presence of an antibody. The 60 and 45 kDa proteins also contain peptides which show identity to peptides of the 60 kDa chaperonin of *Pseudomonas fluorescens* and elongation factor Tu of *Yersinia pestis* (43 kDa) respectively which is involved in peptide bond synthesis. The 32 kDa protein contains peptides which show identity to peptides of the outer major protein of *Serratia marcescens* (37 kDa) and peptides of the Ig lambda chain of *Gallus gallus.* All homologous peptides are shown in the Appendix.

Table 3.1 Summary of MS analysis of possible affinity purified IBDV receptor proteins

Affinity purified protein molecular mass (kDa)	Proteins detected by >2 peptides*	Species	No. of peptides recognised	Function
70	Ig gamma chain	*Gallus gallus*	13	The heavy chain of IgY
60	Ig gamma chain	*Gallus gallus*	10	The heavy chain of IgY
	60 kDa chaperonin (Cpn60)	*Pseudomonas fluorescens*	3	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions
45	Ig gamma chain	*Gallus gallus*	8	The heavy chain of IgY
	Elongation factor-Tu	*Yersinia pestis*	5	Involved in peptide bond synthesis
32	Ig gamma chain	*Gallus gallus*	3	The heavy chain of IgY
	Outer major protein (OmpA)	*Serratia marcescens*	5	Serves as a receptor for a number of T-even like phages Also acts as a porin with low permeability that allows slow penetration of small solutes
	Ig lambda chain	*Gallus gallus*	3	The light chain of IgY

***peptides have been identified with at least 95% confidence**

3.5 DISCUSSION

The identification of virus receptors is crucial to the understanding of virus pathogenesis and their mechanism of entry into susceptible cells. Even though the IBDV receptor(s) has not been identified, VP2 has been shown to be the receptor binding protein (Yip *et al.*, 2007). The present study was aimed at identifying the proteins which bind to IBDV and VP2 and may therefore represent the IBDV receptor(s). In the previous chapter, VP2 was recombinantly expressed and purified. In work described in the current chapter, IBDV was isolated from infected bursal tissue and the isolated virus was allowed to propagate in Vero cells and purified. Purified IBDV or VP2 was used in a virus overlay protein-binding assay (VOPBA) to identify possible receptor proteins. In addition VP2 binding proteins of the bursae were affinity purified and analysed by mass spectrometry.

The bursa of Fabricius is the target organ for IBDV, therefore the first part of the current study required the isolation of plasma membrane proteins from the bursae to identify proteins specifically binding to VP2 or IBDV. Two methods were used to isolate the membranes. The first method is a protocol described by Nieper and Muller (1998) which uses high speed centrifugation washes and the second method used a ProteoPrep® membrane extraction kit (Sigma-Aldrich-Fluka, Steinheim, Germany) based on protocols described by Molloy *et al.* (1998) and Herbert *et al.* (1998). Both methods were successfully applied to isolate two proteins of 40 and 43 kDa which is comparable to the results described by Nieper and Muller (1998). The extraction kit, however, produced a higher yield of membrane proteins. Using an extraction kit provides many advantages which include expediency, increased reproducibility and quality of results (Tan and Yiap, 2009) which could explain the higher yield obtained using the extraction kit. Plasma membrane proteins were therefore successfully isolated and used in VOPBA experiments.

A VOPBA was used to identify IBDV binding proteins and possible IBDV receptor(s) on the plasma membranes isolated from the bursae. The VOPBA has been successfully used to identify many virus receptors such as the mouse hepatitis virus A59 (Dveksler *et al.*, 1993), porcine epidemic diarrhoea virus (Oh *et al.*, 2003) and adenovirus (Trauger *et al.*, 2004). In a conventional VOPBA, whole virus is used to probe plasma membranes blotted onto nitrocellulose; therefore the second part of the current study required the isolation of IBDV. The virus was successfully purified

from infected bursae which was confirmed by the presence of viral proteins VP2, VP3 and VP4 at their expected molecular masses. Becht (1994) reported that VP2 and VP3, the major structural proteins, are present in equal amounts in IBDV isolates. This correlates well to the current study as VP2 and VP3 were found to be the predominant proteins present in the virus preparation analysed by SDS-PAGE. Isolated IBDV was propagated in Vero cells which are derived from normal adult African green monkey kidney cells. Cytopathic effects were observed 96 h post-infection. The IBDV particles were thereafter purified and used in a conventional VOPBA.

A conventional VOPBA using IBDV particles and a modified VOPBA using VP2 (the receptor binding protein) were performed. In addition, the VOPBA experiments were designed to include chicken anti-VP2 antibodies or chicken anti-VP2 peptide antibodies. Varying results were obtained for each of the VOPBA experiments. The VOPBAs incubated with anti-VP2 peptide antibodies provided inconclusive results since the anti-peptide antibodies did not detect any proteins in the plasma membrane isolates which IBDV or VP2 specifically bound to. It is unclear why the chicken anti-VP2 peptide antibodies did not detect any proteins. One of the advantages of using an anti-peptide antibody is that it can be directed to a specific target sequence and therefore promote specificity (Adams *et al.*, 1997). This advantage, however, can also be seen as a shortfall since only a single epitope in the target protein is recognised while an antibody to the whole protein can recognise multiple epitopes in a target protein. According to the 3D structure of VP2, the epitope recognised by the chicken anti-VP2 peptide antibodies is located on the surface and spans amino acids 26 to 39 while the amino acids which are responsible for tissue culture adaptation and believed to bind directly with the cellular receptor(s) are 253, 279 and 284 (Yip *et al.*, 2007). However, binding of chicken anti-VP2 peptide antibodies to VP2 or IBDV depends on how or with which orientation they bind to membrane proteins. It is therefore possible that the VOPBAs incubated with chicken anti-VP2 peptide antibodies did not detect any proteins because IBDV or VP2 epitopes for recognition were not accessible for binding.

The recombinant VP2 specifically bound to a 70 and a 32 kDa protein and IBDV specifically bound to a 32 kDa protein which was detected with chicken anti-VP2 antibodies in a VOPBA. Previous studies have reported the interaction of IBDV with

proteins of 70 (Setiyono *et al.*, 2001) and 32 kDa (Edwards, 2000) which is comparable to the current study. Setiyono *et al.* (2001) used a highly virulent strain of IBDV propagated in LSCC-BK3 cells and the interaction of IBDV with the 70 kDa protein was observed in a VOPBA. In addition to the 70 kDa protein, detection of an 82 and a 110 kDa protein was reported (Setiyono *et al.*, 2001) which was not observed in the current study. The Setiyono *et al.* (2001) study used plasma membranes isolated from LSCC-BK3 cells in a VOPBA whereas the current study used plasma membranes isolated from the bursae which may therefore explain the different results. Edwards (2000) observed the interaction of IBDV with a 32 kDa protein by reversibly cross-linking IBDV to potential receptor molecules on bursal cells via 2-iminothiolane. In this method, virus is allowed to bind whole cells via the receptor(s) which are then reduced and cross-linked to virus via 2-iminothiolane. The cell membranes are disrupted and potential receptor(s) purified using density gradient centrifugation. This method of reversibly cross-linking virus to potential receptor(s) on cells represents the natural state of viral attachment therefore improving the accuracy of the results.

In a study conducted by Nieper and Muller (1996), two proteins of the bursal plasma membrane were observed binding to IBDV with molecular masses of 40 and 46 kDa in a VOPBA. A 40 and 46 kDa protein was not observed binding to IBDV or VP2 in the current study. An attenuated strain of IBDV was used in the Nieper and Muller (1996) study, which requires multiple passages before attenuation in CEF cells. Tissue culture adaptation and attenuation of IBDV alters amino acid residues in the VP2 amino acid sequence (Yamaguchi *et al.*, 1996b, van Loon *et al.*, 2002, Kwon and Kim, 2004) which is thought to engage directly with the cellular receptor (Coulibaly *et al.*, 2005). The attenuated strain may therefore bind different proteins on the surface of plasma membranes compared to the non-attenuated strain of IBDV used in the current study and consequently producing different results. Although the VOPBA has been successfully used to determine viral receptors (Cao *et al.*, 1998a, Li *et al.*, 2003), the method has some limitations. Purified virus is required to interact with linearised polypeptides, assuming the interaction between virus and protein is non-conformational. Therefore, in addition, affinity purification of VP2-binding proteins from bursal plasma membranes was used to confirm and compare the results obtained in the VOPBA.

The VP2 binding proteins were isolated from bursal plasma membranes using a VP2-coupled affinity matrix. Four proteins with molecular masses of 70, 60, 45 and 32 kDa were purified. Isolation of the 70 and 32 kDa proteins provided positive comparable results since proteins with these molecular masses were also detected in the VOPBA. In the VOPBA method, the plasma membrane proteins were denatured before separation by SDS-PAGE whereas during the affinity purification, proteins maintain their conformation. The purification of additional proteins not detected in the VOPBA was therefore expected. As previously mentioned, a study conducted by Nieper and Muller (1996) reports on the interaction of a 46 kDa protein with IBDV which is speculated to be the 45 kDa protein purified in the current study. Reports, however, could not be established which describe a 60 kDa IBDV binding protein. To identify the proteins, the protein bands in the SDS-PAGE gel were excised and in-gel digested with trypsin and the peptides analysed by mass spectrometry. The mass spectra were compared with the Uniswiss 2011 protein database which identified several peptides common to a few proteins.

A high degree of sequence identity was found with the immunoglobulin (Ig) gamma chain of IgY in all the proteins purified. It is unlikely that this was a contamination of IgY which was previously purified on the same column (Chapter 2) since the VP2-column was thoroughly washed and regenerated. A solution of 1 M sodium hydroxide was used to clean the column which is commonly used to successfully remove proteins bound to chromatography columns (Block, 1991, Hale *et al.*, 1994). Although IgY is mainly a circulatory antibody, it has been observed expressed on the surface of bursal B cells (Chen *et al.*, 1982, Sharma, 1990) much like its mammalian homologue IgG (Lafrenz *et al.*, 1986). IgG and IgY are similar in that they both possess two heavy and two light chains, have similar sedimentation coefficients of about 7S and play the same biological role of providing defence against infectious agents (Larsson *et al.*, 1993). In addition, the Ig gamma chain of IgY identified in the current study belongs to the immunoglobulin superfamily. Therefore this Ig receptor-like protein represents a good candidate as the IBDV receptor(s) since many viruses have been shown to exploit proteins of the immunoglobulin superfamily to gain entry into host cells (Dermody *et al.*, 2009, Perez *et al.*, 2009). A few examples include CD4, the HIV receptor, which has four immunoglobulin domains (Dalgleish *et al.*, 1984), Poliovirus receptor, CD155 which has three immunoglobulin domains (Mendelsohn *et al.*, 1989) and the Reovirus

receptor, JAM-A which has two immunoglobulin domains (Barton *et al.*, 2001, Prota *et al.*, 2003). The chicken B-cell expresses several cell surface proteins which belong to the immunoglobulin superfamily such as ICAM-1 which is an adhesion molecule which binds B-cells to other cells (Huang and Springer, 1995) and CD80 which binds to T-cells producing co-stimulatory signals which activate B-cells (Peach *et al.*, 1995). Therefore it is hypothesised that this Ig receptor-like protein belonging to the immunoglobulin superfamily may possibly form part of the IBDV receptor which is expressed on the surface of B-cells.

Peptides of the 60 kDa affinity purified protein also demonstrated sequence identity with peptides of the 60 kDa chaperonin of the plant bacterium *P. fluorescens* while the 45 kDa protein demonstrated identity with the 43 kDa elongation factor of *Y. pestis.* These two results were difficult to explain since these are both bacterial proteins. Interestingly a study conducted in our laboratory by Edwards (2000) reported on a 32 kDa protein observed to interact with IBDV, which demonstrated sequence identity with the root adhesin from *P. fluorescens.* A part of the 32 kDa protein's gene sequence was successfully amplified by PCR and RT-PCR using chicken DNA and RNA respectively, which confirmed the protein to be encoded by the chicken genome (Edwards, 2000). Similarly, it is suggested that the 60 and 45 kDa proteins identified in the current study are encoded by the chicken genome which has not yet been completely sequenced. In order to test this theory, PCR and RT-PCR should be performed on the identified sequences using degenerate primers.

The 32 kDa protein identified in the current study shares common peptides with the outer major protein of *S. marcescens* and the Ig lambda chain. Again it is suggested that the 32 kDa protein is encoded by the chicken genome and shares common peptides with the outer major protein of *S. marcescens.* Interestingly though, is the function of the outer major protein which serves as a receptor for several T-even like phages and also acts as a porin (Malouin *et al.*, 1990). The outer major protein therefore represents a good candidate as a receptor binding protein although further analysis is required to ensure it is a protein which is encoded by the chicken genome. Sequence identity of the 32 kDa affinity purified protein to the Ig lambda chain further increases the evidence that an Ig receptor-like protein may form part of the IBDV receptor. A study conducted by Luo *et al.* (2010) demonstrated that the

lambda light chain of surface IgM specifically interacts with IBDV. This was performed by recombinantly expressing the lambda light chain and analysing its ability to bind IBDV in a VOPBA (Luo *et al.*, 2010). Therefore it is again observed that the 32 kDa protein represents a good candidate as an IBDV receptor or plays a role in IBDV entry.

In summary, several proteins have been identified which represent good candidates as forming part of the IBDV receptor(s). Further studies are necessary to demonstrate and confirm their involvement in IBDV infection which could lead to the design and production of antiviral inhibitors which target viral entry. These results and any future studies which could be built on from the current study are discussed in the following chapter.

CHAPTER 4

GENERAL DISCUSSION

Infectious bursal disease virus (IBDV) is a Birnavirus causing infectious bursal disease (IBD), a highly contagious immunosuppressive disease which affects young chickens (Kibenge *et al.*, 1988). IBDV infects the immature antibody producing B-cells of the bursa of Fabricius (Kibenge *et al.*, 1988), depleting the bursa of B-cells as replication of viral particles takes place and bursal cells become atrophic (Tanimura and Sharma, 1997, Sharma *et al.*, 2000). Infected chickens, as a result, develop a compromised immune system which leaves them susceptible to other opportunistic pathogens which in turn increases their mortality rate (Saif, 1991). In addition, the disease is highly contagious, resistant to inactivation and spreads rapidly through contaminated feed and water (van den Berg *et al.*, 2000). Consequently IBD has caused major economic losses in the poultry industry (Muller *et al.*, 2003).

The current control strategy for IBD is vaccination (Muller *et al.*, 2012). Although there are various IBD vaccines being used such as subunit vaccines, IBDV immune complex vaccines and live viral vector vaccines, the success of the vaccines is dependent on various factors. These include presence of maternally derived antibodies, the age of chickens and time period during which vaccination is administered, vaccination strains and epidemiological field conditions (Rautenschlein *et al.*, 2005). Maternally derived antibodies in young chickens (Corley and Giambrone, 2002) and the continuous emergence of new virulent IBDV strains (Cao *et al.*, 1998b) limit vaccine effectiveness and in addition some vaccines have been shown to cause moderate to severe bursal atrophy (Rautenschlein *et al.*, 2005). Therefore it is important to focus on new strategies to control the disease.

Virus attachment to receptor proteins, which assist in entry into host cells, is a critical step in the virus life cycle (Smith and Helenius, 2004). Development of antiviral agents which inhibit entry is a novel alternative strategy to control the disease. The identity of the receptor binding proteins of the virus and the receptors on target cells are required for the development of antiviral agents. Viral entry, however, of many non-enveloped viruses is at present not fully resolved as it is a complex process which utilises penetration to gain access into host cells. Virus must

first initiate interaction with receptor(s) and/or co-receptor(s) which activates a series of events which ultimately allows successful infection of host cells. Therefore, in order to produce antiviral agents which target viral entry, in depth knowledge of receptor(s) and/or co-receptor(s) and the comprehensive understanding of the virus mechanism of entry is required. VP2 is the receptor binding protein of IBDV (Yip *et al.*, 2007), however, the receptor(s) on B-cells used for IBDV entry have not been identified although various studies have been conducted. Nieper and Muller (1996) demonstrated the ability of attenuated IBDV to bind a 40 and 46 kDa protein on CEF cells and Ogawa *et al.* (1998) reported that the IBDV receptor is an N-glycosylated protein associated with the expression of B-cell surface IgM. Later, Setiyono *et al.* (2001) demonstrated a very virulent strain of IBDV binding to three proteins with molecular masses of 70, 80 and 110 kDa on LSCC-BK3 cells while Lin *et al.* (2007) reported that chicken heat shock protein 90 (Hsp90) forms part of and is required for IBDV entry into DF-1 cells. Although extensive research has been conducted on identifying the IBDV receptor(s), it is yet to be conclusively determined.

The aim of this study was to determine possible IBDV receptors on host cells of the bursa of Fabricius by different experimental techniques through the use of the receptor binding protein, VP2. Previous studies used different IBDV susceptible cell lines to assist in identifying the IBDV receptor(s). Susceptible cells used were CEF cells (Nieper and Muller, 1996, Ogawa *et al.*, 1998), LSCC-BK3 cells (Setiyono *et al.*, 2001) and DF-1 cells (Lin *et al.*, 2007). The current study, however, used a different approach. Plasma membrane proteins from the bursa of Fabricius and VP2, the receptor binding protein, were used. First, a conventional and modified VOPBA incubated with IBDV or VP2 respectively was used with either chicken anti-VP2 antibodies or chicken anti-VP2 peptide antibodies to determine IBDV binding proteins. Secondly VP2-binding proteins from the bursal plasma membrane were affinity purified on a VP2-coupled affinity matrix and analysed by mass spectrometry. To do this the VP2 protein was recombinantly expressed, affinity purified and used to prepare a VP2-coupled affinity matrix. In addition recombinant VP2 and peptides identified from the VP2 amino acid sequence were used to raise polyclonal chicken anti-VP2 antibodies and chicken anti-VP2 peptide antibodies to assist in identifying IBDV binding proteins in a virus overlay protein binding assay (VOPBA).

Since the main aim required the use of VP2 to assist in identifying the IBDV receptor(s), large quantities of recombinant VP2 was required. The VP2 coding sequence was previously cloned into pGEX-4T-1 and pET-32a expression vectors and transformed into *E. coli* cells in our laboratory. Therefore expression of the VP2 protein was attempted in the *E. coli* expression systems. Auto-induced expression of VP2 using the pET-32a *E. coli* expression system was successful since VP2 expressed as a 64 kDa and a high molecular mass His-tagged protein. The high molecular mass proteins were believed to be VP2 after mouse anti-His tag monoclonal antibody and later, chicken anti-VP2 antibodies detected them in a western blot. Furthermore, reduction of the high molecular mass proteins and analysis by reducing SDS-PAGE after electro-elution saw the proteins run as a 64 kDa protein band (the expected size of the VP2 fusion protein), as well as towards the top of the gel. Expression of VP2 in the pET-32a *E. coli* system, however, expressed as insoluble inclusion bodies despite subsequent decreases in expression temperature which is known to increase solubility of expressed protein (Schein and Noteborn, 1988). Purification of large enough quantities of the insoluble VP2 was a difficult task. Affinity chromatography of recombinant VP2 via the His-tag under denaturing conditions saw VP2 co-purify with other contaminating bacterial proteins. In addition purification under denaturing conditions was considered unfavourable since correctly folded VP2 was required for preparation of a VP2-coupled affinity matrix. On-column refolding of recombinant VP2 also via the His-tag was therefore preferred and was successfully used to purify recombinant VP2. Purification though produced low yields of pure recombinant VP2 and large amounts were observed in the unbound and wash fractions during on-column refolding. The wash fractions containing VP2 were therefore retained and VP2 successfully purified from these samples using ion-exchange chromatography. Since purifying large enough yields of recombinant VP2 was difficult alternative expression systems were considered.

The *Pichia pastoris* yeast expression system was considered a favourable option since expressed proteins are predominantly secreted allowing for ease of purification. Additionally, VP2 has been previously expressed in this system (Pitcovski *et al.*, 1996, Wu *et al.*, 2005, Villegas *et al.*, 2008). The VP2 coding sequence was therefore sub-cloned into the pPIC9 expression vector and integrated into the *P. pastoris* genome. The VP2 protein was successfully expressed as a 47 kDa and high molecular mass protein which was concentrated and purified using

three-phase partitioning. Molecular exclusion chromatography was used to further purify VP2 from contaminating proteins and successfully separated the high molecular mass VP2, but not the 47 kDa VP2 from other proteins. Large enough yields of purified recombinant VP2 in the form of incorrectly folded or high molecular mass proteins was therefore obtained for further use. Although the high molecular mass proteins were sufficiently detected by chicken anti-VP2 peptide and chicken anti-VP2 antibodies to identify them as VP2 proteins, sequencing of the recombinant protein for further identification was not carried out. Sequencing of the high molecular mass proteins could further corroborate and confirm this observable result. Recombinant VP2 expressed in the *E. coli* expression system was used to raise polyclonal chicken anti-VP2 antibodies while VP2 expressed in the *P. pastoris* expression system was used to produce a VP2-coupled affinity matrix for the purification of chicken anti-VP2 antibodies and VP2-binding proteins of the bursal plasma membrane. Additionally two peptides were selected for the production of chicken anti-VP2 peptide antibodies. The idea was to compare and determine the chicken anti-VP2 and chicken anti-VP2 peptide antibodies' ability to bind VP2 or IBDV in a VOPBA.

Polyclonal chicken anti-VP2 antibodies were successfully produced as evidenced by the high antibody titres obtained which therefore suggests the recombinant VP2 is able to maintain its immunogenic properties as native VP2. Although comparably low antibody titres were observed for antibodies produced against one of the peptides (CKMVATAbuDSSDR)), antibodies against a second peptide (ASIPDDTLEKHTLRC) were relatively high and therefore, overall, chicken antibodies were successfully produced against recombinant VP2 and against a peptide. To determine IBDV-binding proteins in a conventional and modified VOPBA, plasma membrane proteins were isolated from the bursa of Fabricius of healthy chickens and IBDV was isolated from the bursa of Fabricius of IBDV infected chickens. The isolated IBDV was propagated in Vero cells and purified for use in a conventional VOPBA while recombinant VP2 was used in a modified VOPBA. Chicken anti-VP2 antibodies or chicken anti-VP2 peptide antibodies were used to detect IBDV-binding proteins and possible IBDV receptor(s). In addition, VP2 binding proteins and possible IBDV receptor(s) of the bursal plasma membranes were affinity purified on a VP2-coupled affinity matrix and identified using mass spectrometry.

The findings presented here are comparable with previous studies, however, new data was obtained which offers further insight into the identity of the IBDV receptor(s). Given that this study used recombinant VP2 and plasma membrane proteins of the bursa of Fabricius, novel interactions were expected. Four IBDV-binding proteins were identified which may represent the putative receptor(s) and/or co-receptor(s) for IBDV. The most significant IBDV binding proteins identified in the current study were two proteins with molecular masses of 70 and 32 kDa. In both the affinity purification and VOPBA experiments a 70 and a 32 kDa protein was purified and binding of VP2 and IBDV observed. Interestingly, a study conducted by Setiyono *et al.* (2001) reports that a 70 kDa plasma membrane protein of LSCC-BK3 cells specifically bound to IBDV in a VOPBA. Moreover, a study conducted previously in our laboratory reports that a 32 kDa protein was observed interacting with IBDV by reversibly cross-linking IBDV to potential receptor molecules on bursal cells via 2-iminothiolane (Edwards, 2000). Therefore the results of the present study are in agreement with those of previous reports. No additional proteins were observed binding in the VOPBA experiments while affinity purification of VP2-binding proteins of the bursal plasma membrane also produced a 60 and a 45 kDa protein. Setiyono *et al.* (2001) also reported that a 82 and a 110 kDa plasma membrane protein bound to IBDV in the VOPBA which was not observed in the present study, however, a study conducted by Nieper and Muller (1996) reported that IBDV bound to two proteins of 40 and 46 kDa on bursal plasma membranes. Here we speculate that the 45 kDa protein purified in the current study may be the 46 kDa protein identified by Nieper and Muller (1996). Tandem mass spectrometry was performed on the four affinity purified proteins to assist in further identification of these VP2 binding proteins and possible IBDV receptor(s).

Mass spectrometry analysis identified a diverse group of proteins which share common peptides with the affinity purified proteins. These proteins included the Ig-gamma chain of IgY, the Ig-lambda chain, outer major protein of *S. marcescens*, the 60 kDa chaperonin of *P. fluorescens* and elongation factor-Tu of *Y. pestis.* Identification of the Ig gamma-chain (belonging to the immunoglobulin superfamily) suggest that IgY is a potential candidate as the IBDV receptor. This is a plausible theory since many viruses have been shown to exploit proteins of the immunoglobulin superfamily to gain entry into host cells such as the rhinovirus which exploits ICAM-1 (five immunoglobulin domains) to gain access into respiratory epithelial cells (Tomassini *et al.*, 1989, Dermody *et al.*, 2009) and HIV which exploits

CD4 (four immunoglobulin domains) to gain access into T-cells (Dalgleish *et al.*, 1984, Dermody *et al.*, 2009). In addition, although IgY is identified mainly as a circulatory antibody, a small percentage of bursal B-cells express surface IgY (Chen *et al.*, 1982, Sharma, 1990). Interestingly, some bursal B-cells which are IgY^+ express surface IgM (Kincade and Cooper, 1971, Chen and Cooper, 1987) and it is believed that surface IgM-bearing B-cells are the target cells for IBDV infection (Hirai and Calnek, 1979, Ogawa *et al.*, 1998). Therefore, overall, IgY is a good candidate as the receptor/co-receptor of IBDV. The Ig-lambda chain of IgM has been shown to specifically interact with IBDV in a VOPBA (Luo *et al.*, 2010), therefore identifying the Ig-lambda chain is in agreement with previous studies.

Mass spectrometry analysis also identified bacterial proteins which have common peptides with the four affinity purified proteins. Although the chicken genome has been extensively studied and drafts of the genome sequence have been presented (ICGSC, 2004) several genes have not been annotated. It is therefore likely that the proteins identified in the current study do in fact belong to the chicken genome. What is interesting about outer major protein of *S. marcescens* is that it is a membrane protein and functions as a cell surface receptor for T-even like phages (Braun and Cole, 1984). The 60 kDa chaperonin of *P. fluorescens* and elongation factor-Tu of *Y. pestis*, however, are not membrane bound proteins and their functions are to promote the correct folding of polypeptides and assist in peptide bond synthesis respectively. Further investigation is therefore required to determine if the bacterial proteins identified do in fact belong to the chicken genome and once this is established, their role in IBDV entry can be further resolved.

Various studies can be developed from the data and sequence identities obtained in the current study. First, since IgY was identified as a possible receptor for IBDV infection, cloning and recombinantly expressing the Ig-gamma chain of IgY and analysing its ability to bind IBDV in a VOPBA could further elucidate this notion. Furthermore, since the sequences of the proteins have been identified, anti-peptide antibodies or monoclonal antibodies against the peptide sequences can be produced and used in cell culture to determine their ability to inhibit IBDV entry. Second, a study is required which can determine whether the bacterial proteins identified are in fact proteins which belong to the chicken genome. Successful PCR and RT-PCR of the identified peptides using chicken DNA or RNA degenerate

primers would provide supportive evidence. Once this is established, their roles as an IBDV receptor or co-receptor can be further determined.

In conclusion, utilising the receptor binding protein, VP2 to identify IBDV-binding proteins was successfully achieved. Four potential proteins were identified using VOPBA experiments and affinity chromatography which were further characterised by mass spectrometry. The results obtained from the current study provide a basis from which to work on in identifying the IBDV receptor(s). Taken as a whole, identifying the proteins in this study as the IBDV receptor will help in the design and production of antiviral agents which target and obstruct IBDV entry into bursal B-cells and therefore assist in the control of IBDV infection.

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APPENDIX

Peptide sequences identified by mass spectrometry

Table 1 Peptide sequences of the 70 kDa affinity purified protein

Protein Identified	Homologous Peptide Sequences
Ig gamma chain	SFVCSAAPGGALLK VDPVPPVAPEVQVLH AIPPSPGELYISLDAK SAVPVSTQDWLSGER TVQHEELPLPLSK NTGPTTPPLIYPFAPHPEELSLSR AVPATEFVTTAVLPEERTANGAGGDGDTFFVYSK

Table 2 Peptide sequences of the 60 kDa affinity purified protein

Protein Detected	Homologous Peptide Sequences
Ig gamma chain	GHGTEVIVSSASPTSPPR SFVCSAAPGGALLK VDPVPPVAPEVQVLH SAEVEWLVDGVGGLLVASQSPAVR VNVSGTDWR VRHPATNTVVEDHVK AIPPSPGELYISLDAK SGNLRPDPM SAVPVSTQDWLSGER AVPATEFVTTAVLPEER
60 kDa chaperonin (Protein Cpn60) (groEL protein)	AVAAGMNPMDLK EMLPVLEAVAK SALQAASSIGGLILTTEAAVADAPK

Table 3 Peptide sequences of the 45 kDa affinity purified protein

Protein Detected	Homologous Peptide Sequences
Ig gamma chain	GTEVIVSSASPTSPPR SAAPGGALLK SPASAEVEWLVDGVGGLLVASQSPAVR AVPATEFVTTAVLPEERTANGAGGDGDTFFVYSK
Elongation factor - Tu	ELLSAYDFPGDDLPVVR ALEGEAEWEAK AIDKPFLLPIEDVFSISGR VGEEVEIVGIK AGENVGVLLR

Table 4 Peptide sequences of the 32 kDa affinity purified protein

Protein Detected	Homologous Peptide Sequences
Ig gamma chain	VSGTPVKLSFVR VRHPATNTVVEDHVK AVPATEFVTTAVLPEER
Immunoglobulin lambda chain	ALTQPASVSANLGGTVK SPGSAPVTVIYDNDKR
Outer major protein	SDVLFNFNK AEGQQALDQLYTQLSSMDPKDGSVVVLGYTDAVGSDQYNQK AQSVVDYLVSK GIKDVVTQPQG