

**Expression and characterization of full length and truncated versions
of major outercapsid protein VP2 of bluetongue virus in bacterial and
insect cells**

by

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DECLARATION

I, the undersigned, hereby declare that the dissertation submitted herewith for the degree *Magister Scientiae* to the University of Pretoria contains my own independent work and has not been submitted for any degree at any other university.

Ritesh Mewalal

July 2010

“Of course there is no formula for success except perhaps
an unconditional acceptance of life and what it brings”

...Arthur Rubinstein
(1886 - 1982)

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LIST OF ABBREVIATIONS

A	ampere
aa	amino acid
AHSV	African horsesickness virus
ANET	Acetate (pH 5.0) sodium chloride, EDTA, Triton X-100 buffer
AcMNPV	<i>Autographa californica</i> multiple nuclear polyhedrosis virus
bp	base pairs
BT	bluetongue disease
BTV	bluetongue virus
BTV(10)	bluetongue virus, serotype 10
C	Celsius
Cfu	colony forming unit
°C	degrees Celsius
dH ₂ O	distilled water
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
et al.	<i>et alibi</i>
e.g.	example
g	grams
GPA	glycoporin A
i.e.	that is
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IgY	immunoglobulin class Y
hrs	hours

kDa	kilo Daltons
LB-broth	Luria-Bertani broth
M	molar
mA	milliamps
M _k	mock
ml	millilitre
M.O.I	multiplicity of infection
M	molecular weight
MWM	molecular weight marker
N	native protein
ng	nanograms
nm	nanometers
NS	non-structure
OD ₆₀₀	optical density at 600 nm
OIE	Office International des Epizooties
ORF	open reading frame
P	pellet
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
p.i.	post-infection
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
Sf	<i>Spodoptera frugiperda</i>
STE	Sodium Chloride–Tris–EDTA

S	supernatant
TAE	tris-acetate-EDTA
TE	tris-EDTA
TF	trigger factor
TEMED	N,N,N',N'-tetramethylethylene diamine
TNET	Tris-HCl (pH 8.0) sodium chloride, EDTA, Triton X-100 buffer
Tris	Tris hydroxymethyl aminomethane
UHQ	ultra high quality water
μl	microlitres
UV	ultra violet
V	volts
v	volume
VIBs	viral inclusion bodies
VLPs	viral-like particles
VP	viral protein
WT	wild type
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

Expression and characterization of full length and truncated versions of major outercapsid protein VP2 of bluetongue virus in bacterial and insect cells

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SUMMARY OF THIS DISERTATION

The spread of bluetongue virus (BTV) to previously disease-free regions which prohibit the use of the current BTV live-attenuated vaccine has highlighted the need for a new generation of vaccines (Ferrari, De Liberato et al. 2005; Veronesi, Hamblin et al. 2005). Subunit vaccines are one of the attractive alternative strategies. Subunit vaccines against BTV would target the outercapsid protein VP2, the main neutralization-specific antigen (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). A subunit vaccine based on the use of BTV-VP2 may be achieved by either using VP2 by itself or by means of virus-like particles (VLPs) on which VP2 proteins are exposed. In VLPs, the VP2 is co-expressed with other capsid and core proteins to form a particle that resembles the intact BTV. The BTV-VLP vaccine strategy is advantageous since it presents the neutralizing epitopes of more than one viral protein in a more authentic manner as found on the virus itself (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). However there are difficulties associated with large scale production and a decrease in the stability of the particles over time (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). Studies have already demonstrated the vaccine potential of BTV-VP2 by itself (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). However if BTV-VP2 is to be used by itself as a single subunit vaccine, it is important that the protein is expressed under conditions where it is correctly folded and soluble. Solubility refers to the capacity of the expressed antigen to fold into an ordered tertiary structure that authentically exposes the neutralizing epitopes to the immune system (Dinner, Sali et al. 2000; Dobson 2003). However non-native interactions within and between *in vitro* synthesized viral proteins such as BTV-VP2 often leads to protein aggregation or insolubility. The immune response against aggregated or insoluble proteins is generally very poor. This problem of aggregation and insolubility may be alleviated to an extent by generating truncated versions of the protein from which hydrophobic regions that promote aggregation have been deleted leaving only the major neutralizing epitopes of the antigen (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

The focus of the research presented in this dissertation was to evaluate the solubility of full-length BTV(10)-VP2 and truncated versions thereof after expression in a prokaryotic and baculovirus-Sf9 expression system. The full-length BTV(10)-VP2 (956 amino acids) gene and genes encoding

truncated versions of BTV(10)-VP2 i.e. BTV(10)-VP2(aa450) (amino acid 1 to 450) and BTV(10)-VP2(aa650) (amino acid 1 to 650) were cloned into the bacterial expression vector pET160-DEST and the baculovirus expression vector pDESTTM8. The C-terminal hydrophobic regions which might contribute to aggregation or insolubility of the protein when expressed *in vitro* were deleted from these truncated BTV(10)-VP2 proteins. The truncated proteins however still contained BTV neutralizing epitopes that were predicted from literature.

The prokaryotic expression of the full-length BTV(10)-VP2 and the other truncated recombinant BTV(10)-VP2 proteins was carried out in *E. coli* BL21 Star DE3 expression strain. The initial pilot expression study confirmed high level expression of the recombinant proteins. The study also revealed that these proteins were insoluble. The optimization of the prokaryotic expression in order to increase the yield of soluble proteins by means of differential inducer concentrations, fermentation temperature and harvesting times did not produce soluble BTV(10)-VP2 and truncated BTV(10)-VP2 proteins. Previous studies have demonstrated the role of L-arginine in the recovery of soluble proteins from aggregation by reversing aggregation (Tsumoto, Umetsu et al. 2003). However in the current study, arginine treatment of the inclusion body and bacterial lysate containing the BTV(10)-VP2 and truncated recombinant proteins did not release soluble proteins. No soluble recombinant BTV(10)-VP2 proteins were detected when the recombinant proteins were expressed in BL21 host cells over-expressing heat-shock proteins (hsps) and chemical chaperones. However when the different recombinant proteins were co-expressed with the molecular chaperones dnaK-dnaJ-GrpE, it resulted in a fraction of soluble recombinant BTV(10)-VP2 proteins. In particular, approximately 50% of the total expressed BTV(10)-VP2(aa450) protein was soluble while approximately 20% of the total expressed BTV(10)-VP2(aa650) and full-length BTV(10)-VP2 were found soluble when co-expressed with dnaK-dnaJ-GrpE chaperones. These recombinant proteins could be eluted from a nickel affinity column further confirming that these proteins are in fact soluble. Interestingly the co-expression of the BTV(10)-VP2(aa450) protein with the above chaperones in combination with chaperones groEL-groES or only groEL-groES did not produce any soluble proteins.

Baculovirus-insect expression of the aforementioned BTV(10)-VP2 recombinant proteins was carried out in *Spodoptera frugiperda* 9 (Sf9) cells. High level expression of the recombinant proteins was confirmed by an initial pilot expression study conducted at 42 hours post infection (p.i.). The pilot study also revealed that the recombinant proteins were insoluble. Arginine treatment of the lysate released a small fraction of soluble BTV(10)-VP2(aa450) and BTV(10)-VP2(ORF) proteins only detectable with immunoblot analysis using the anti-BTV(10) IgY antibodies. The amount of solubilized proteins was however too small to justify the cost associated with this expression system.

CHAPTER 1: LITERATURE REVIEW

1. INTRODUCTION

Orbiviruses are intricate non-enveloped RNA viral particles characterized by the presence of seven structural proteins, a double-stranded RNA genome which is segmented into 10 fragments of varying lengths and replicative potential in both arthropod vector and vertebrate host (Gorman 1979; Roy 1996). Bluetongue virus (BTV) is the archetype member of the genus *Orbivirus* belonging to the family Reoviridae and the etiological agent of the non-contagious bluetongue (BT) disease affecting some species of ruminants e.g. sheep and cattle (MacLachlan 2004; Mertens and Diprose 2004; Bhattacharya, Noad et al. 2007). There are 24 reported serotypes of BTV. The serotype specificity is determined by outer capsid protein VP2 (Huismans, Cloete et al. 1987; Roy 1992). The lack of serotype cross-protection renders vaccine strategies laborious, time consuming and costly (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Savini, Monaco et al. 2004). The current BT vaccination protocol involves a live-attenuated vaccine produced by Onderstepoort Biological Products in South Africa (Dungu, Gerdes et al. 2004).

The recent outbreaks of BT in previously disease free counties such as European counties have placed economic strain on the sheep and cattle industry. In the United States alone, BT causes a loss of approximately \$125 million per annum (Bram, George et al. 2002; Veronesi, Hamblin et al. 2005). It is for this reason that BT is now considered an A-listed Office International des Epizooties (OIE) disease (Schwartz-Cornil, Mertens et al. 2008). These countries prohibit the use of the BT live-attenuated vaccine due to safety concerns (Ferrari, De Liberato et al. 2005; Veronesi, Hamblin et al. 2005). This in-turn advocates the need for a new generation recombinant vaccine which addresses such safety apprehensions. Due to its cost efficiency, effectiveness and safety, subunit vaccinations containing single viral protein preparation or truncated viral proteins have become an attractive vaccine strategy (Dorner, Eibl et al. 1999). In terms of BTV, the serotype determinant VP2, is also the main neutralization-specific antigen and thus an attractive vaccine candidate (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990).

Previous subunit vaccine strategies involving BTV-VP2 have focused on the outer capsid by itself or as part of virus-like particles (VLPs) (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). The BTV VLP vaccine strategy was effective however high level VLP production is difficult and the particle stability is reduced over time (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). If BTV-VP2 is to be used by itself then solubility is an important consideration i.e. the expressed proteins ability to fold into an ordered tertiary

conformation which authentically exposes the neutralizing epitopes to the immune system (Dinner, Sali et al. 2000; Dobson 2003). The immune reaction against aggregated or insoluble proteins is generally poor (Henk Huismans, personal communication, University of Pretoria). Insolubility of expressed antigens may be overcome by expressing truncated versions of a particular antigen such that it contains predominantly the major neutralizing epitopes while lacking hydrophobic regions of the protein which promotes aggregation (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

So while the long-term aim of this project is to develop a subunit vaccine against BTV based on BTV-VP2 and BTV-VP2 derived peptides, this study focused on the evaluation and comparison of the solubility of BTV-VP2 and truncated versions of this protein expressed in a bacterial and baculovirus-Sf9 expression system.

This literature chapter therefore reviews four main aspects, relevant to the focus of this study. The first topic discussed is BT disease. This serves to put into perspective the severity of the disease and the negative implications on animals and industry due to the rapid spread of the virus. This substantiates the need for an effective vaccine strategy. In the second discussion topic, BTV is dissected in terms of structural and molecular biology with an emphasis placed on the outercapsid protein VP2. It is necessary to have a complete understanding of the structural organization of the virion as well as the biological importance of capsid in order to rationalize a BTV-VP2 based vaccine. The third discussion topic is *in vitro* protein expression. This section gives an overview of native protein folding and the implications of mimicking this *in vitro*. Expression systems as a tool for foreign protein synthesis is then discussed highlighting the use of bacterial and baculovirus-Sf9 expression systems for the purposes of this study. The fourth section discusses vaccination strategies by criticizing the various methods. Under this section, there is a focus on advantages and disadvantages of a subunit vaccination strategy and more specifically a BTV-VP2 subunit vaccine approach. This in-turn is weighted against the pros and cons of the current live-attenuated BTV vaccine logically justifying the aims of the current study.

2. BLUETONGUE DISEASE

2.1. History and current global status of BT

BT was first reported in South Africa in the late 19th century subsequent to the supplementation of existing sheep population with fine-wooled sheep from Europe. Due to the apparent cyanosis of the tongue in affected sheep, the disease was aptly named 'Bluetongue'. This was after it was originally referred to as 'malarial catarrhal fever' and 'epizootic catarrh of sheep' (MacLachlan 2004;

MacLachlan, Zientara et al. 2007). Initially, the disease was thought to be confined to African countries until 1941 when an outbreak was confirmed in Cyprus. The global spread of BT post 1941 to countries in America, the Middle East, Asia and Southern Europe has accordingly justified it as an Office International des Epizooties (OIE) listed disease (Mellor and Boorman 1995; Schwartz-Cornil, Mertens et al. 2008). Since 1997, BT has become prevalent within the Mediterranean regions. During this time BTV-8 was detected in Europe and northern European countries. By 2006 BTV-8 was reported in Germany, Belgium and the Netherlands. However it was only after the overwintering period in 2007 that the extent of BTV migration into European countries had become apparent. BTV-8 was reported in Belgium, Germany, France, Netherlands, Luxemburg, Denmark, Switzerland, the Czech Republic and UK with a sum of 40 931 reported cases. Initially, the lack of control over the import of infected sheep and cattle introduced BTV into Italy and Northern Ireland. However the slaughter of the infected animals as well as uninfected sheep and cattle in contact with such animals quickly led to the elimination of this threat. In 2006, BTV-1 was detected in Northern Africa. The same serotype was detected the following year in Spain. The eight serotypes of the virus detected in the USA suggests that there is also a northerly migration of the virus (Mellor and Wittmann 2002; Saegerman, Berkvens et al. 2008; Schwartz-Cornil, Mertens et al. 2008).

2.2. Epidemiology

BTV predominantly affects antelope, deer and sheep with a corresponding morbidity of up to 100% and a mortality of up to 70%. Infections may also be prevalent in cattle, goats and most wild ruminant species however the disease remains asymptomatic with a morbidity of up to 5% and a rare incidence of death. Expression and spread of the disease shows a direct correlation to a) host factors, b) vector factors, and c) virus serotype (Breard, Hamblin et al. 2004; Breard, Sailleau et al. 2004; MacLachlan 2004).

2.2.1. Host factors

Although BTV affects all ruminant species, it seems that susceptibility to the disease is restricted to selected breeds of sheep e.g. fine wool Merino and mutton breeds, deer e.g. white-tailed deer and antelope e.g. pronghorned antelope (Wittmann and Baylis 2000; MacLachlan 2004). This susceptibility is further exploited by the nutritional status, immune status and age of the selected breed. BTV infection of other species of ruminants such as cattle and goats remain sub-clinical. The inherent ability of such animals to resist disease despite the presence of the virus makes them asymptomatic carriers of the virus. The apparent anomaly of the vulnerability of sheep to the BT and resistance of cattle to the disease may be elucidated in terms of the inherent susceptibility of the endothelial cells in sheep. BTV infection of cattle results in activation of the endothelial cells with a

marked immune response including an increase in the expression of vasoactive and inflammatory mediators. This reaction is contrasted in sheep in which there is minimal endothelial cell stimulation (Barratt-Boyes, Rossitto et al. 1995; DeMaula, Leutenegger et al. 2002; MacLachlan 2004). Particles that are erythrocyte associated are protected from the initial immune response. It seems that BTV RNA may be detected 145 days post infection which is similar to ruminant erythrocyte lifespan. Therefore it seems possible that erythrocytes may be the key factor in allowing for cattle to serve a BTV reservoir host (Brewer and MacLachlan 1994).

2.2.2. Vector factors

Due to the lack of maintenance of BTV in its host and the non-contagious nature of the infection, BTV transmission is almost entirely dependent on active adult *Culicoides* biting midges (Diptera: Ceratopogonidae). This in turn suggests that the geographic distribution of the disease is limited to areas occupied by susceptible vertebrates and competent vectors i.e. tropical and subtropical regions of the world, between latitudes of 35°S and 40°N and transmission of BTV occurs chiefly during spring when vector activity begins (Figure 1). Thus in order to fully understand the distribution of BT, it is necessary to consider factors that affects the vector populations itself. Such factors include temperature, precipitation and wind i.e. climatic factors. *Culicoides imicola* has been implicated as the most important vector in the transmission of BTV and AHSV. However this does not exclude *C. obsoletus*, *C. pulicaris*, *C. impunctatus*, *C. nubeculosus* to name a few, which have been found in BTV and AHSV contaminated areas (Wittmann and Baylis 2000).

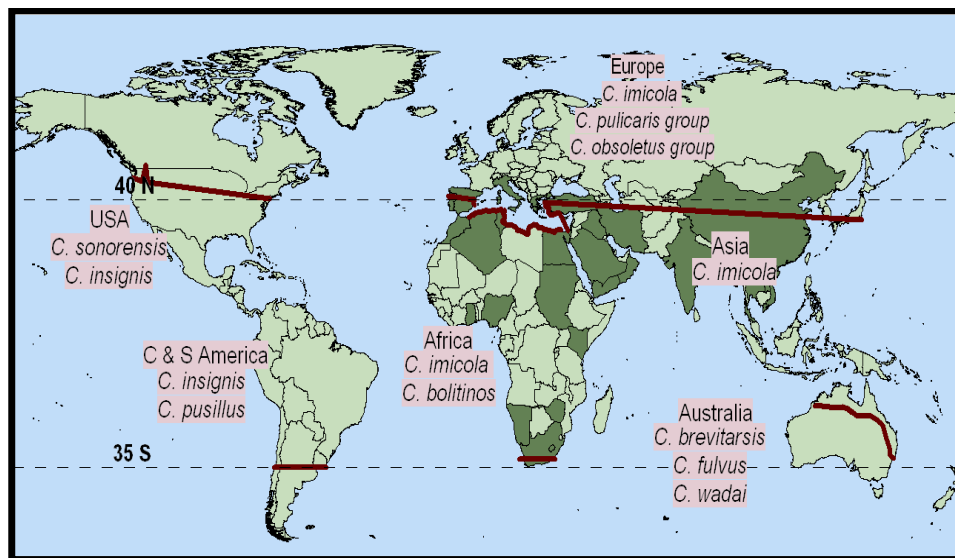


Figure 1. Global distribution of BTV limited to the presence of competent *Culicoides*. Adapted from “Bluetongue virus in Europe: an update after ten years. Are we over the worst?” presentation by P. Mertens (2009)

2.2.3. Virus serotype

Genetic drift and reassortment of viral genes are responsible for the genetic variation existing amongst field strains of the BTV. Reassortment of gene segments and individual genes have been established in both ruminant host and insect vectors post infection (Samal, el-Hussein et al. 1987; Samal, Livingston et al. 1987; MacLachlan 2004). The recent extensive migration pattern of BTV and the quick rate at which this is occurring is alarming since it may favor the generation of new assortments of BTV (Schwartz-Cornil, Mertens et al. 2008). Genetic drift is a direct consequence of the accretion of nucleotide substitutions within BTV genes and occurs by via acquisition and amplification of viral variants within the vector. Such variants are acquired from the quasispecies viral population which are found in the blood of infected host and are the result of founder effect (Bonneau, Mullens et al. 2001). The genetic variability is manifested in the phenotypic differences and virulence amongst BTV strains (MacLachlan 2004).

2.3. Economic Implications of BT

BTV has become a pathogen of economic interest as a result of the substantial loss suffered in the sheep and cattle industry. With regard to the sheep industry such losses are a direct consequence of mortality (mortality of up to 70% in individual flocks) or indirectly in terms of wool growth, reproductive performance, abortions, delayed convalescence and in some instances ram infertility (McKercher, McGowan et al. 1953; Breard, Hamblin et al. 2004). Although the pathogen may not be transmitted to humans, it seems likely that food scares may lead to a decrease in demand for sheep and/or sheep products (Basak, Grimes et al. 1997). BTV also affects the cattle industry since cattle can act as reservoirs for the virus even though they show no clinical signs of the disease. The above combined with the restrictions prohibiting the trade of live animals or animal products from countries known to have the virus to BTV-free countries, means that millions are lost in trade and in diagnostics of sheep and cattle (Saegerman, Berkvens et al. 2008).

2.4. Pathogenesis

As mentioned above abortion, delayed convalescence and death are common occurrences of BTV infection. The infection is characterized by prolonged cell-associated viraemia which persist despite the high titers of viral neutralizing antibodies (Schwartz-Cornil, Mertens et al. 2008). Another indirect but equally severe repercussion of BT is death by secondary bacterial infection (e.g. pneumonia). Initial symptoms of BT in sheep includes a fever of around 42°C, nasal discharge, respiratory problems, oedema of lips and inflammation of the tongue, oral mucosa and coronary band. Severe oedema of the tongue may cause blood flow restrictions (Figure 2). Some of the above symptoms (e.g. nasal discharge, fever, etc.) may occur in cattle, however for the most part infected cattle

remain asymptomatic and serve as reservoirs of the virus (McKercher, McGowan et al. 1953; Pini 1976; Breard, Hamblin et al. 2004).

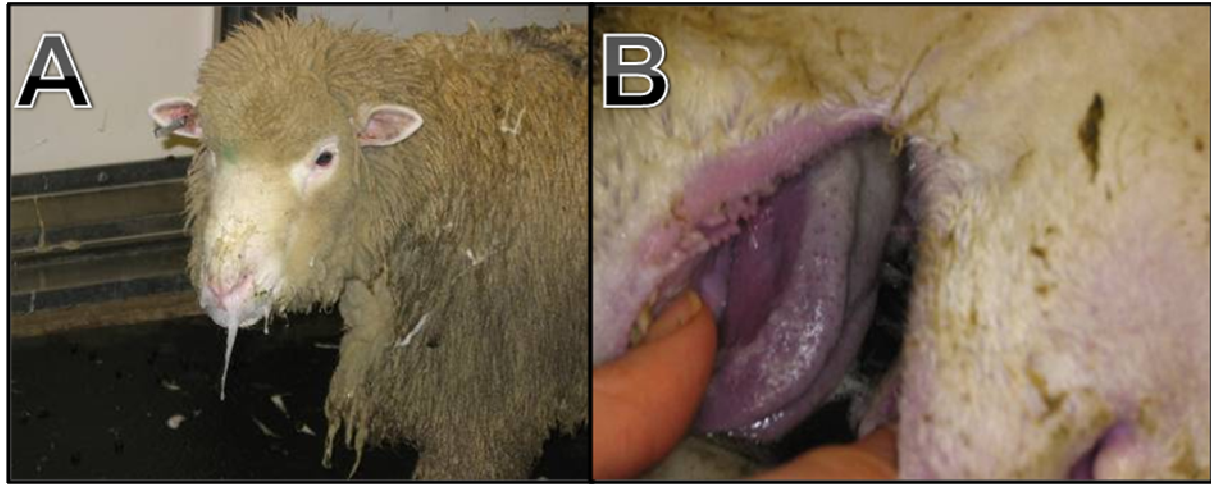


Figure 2. BT disease manifestation in sheep shows a characteristic clear and stringy salivation with nasal discharge (A) and cyanosis of the oral cavity (B). Adapted from “Bluetongue virus in Europe: an update after ten years. Are we over the worst?” presentation by P. Mertens (2009)

3. BLUETONGUE VIRUS (BTV)

BTV is a non-enveloped icosahedral particle with a diameter of 86 nm. The virus has a density of 1.337 g/cm³ and contains a 19 219 base pair double stranded RNA genome which is composed of 57% AU and 43% GC content (Schwartz-Cornil, Mertens et al. 2008). Each viral particle consists of seven structural proteins which are organized into concentric protein shells. The inner-most shell, termed the subcore, is made up of VP3 (103 kDa). The subcore encloses the ten segmented dsRNA molecules as well as the RNA-dependent RNA polymerase (VP1) (150 kDa), the capping enzyme (VP4) (78 kDa) and the helicase (VP6) (36 kDa) (Roy, Fukusho et al. 1988; Stauber, Martinez-Costas et al. 1997; Ramadevi, Burroughs et al. 1998). The VP3 protein layer forms a scaffold for stabilization of the VP7 (38 kDa) trimers which constitutes the viral core. The viral core in turn is surrounded by the outer shell composed of VP2 (110 kDa) and VP5 (59 kDa). The various protein layers of the virion formed by self-assembly are shown in Figure 3 (Roy, Boyce et al. 2009). In addition to the seven structural proteins, the RNA genome also encodes 4 non-structural proteins (NS1, NS2, NS3 and NS3A) observed in virally infected cells (Verwoerd and Huismans 1969; Roy 1992; Huismans, van Staden et al. 2004).

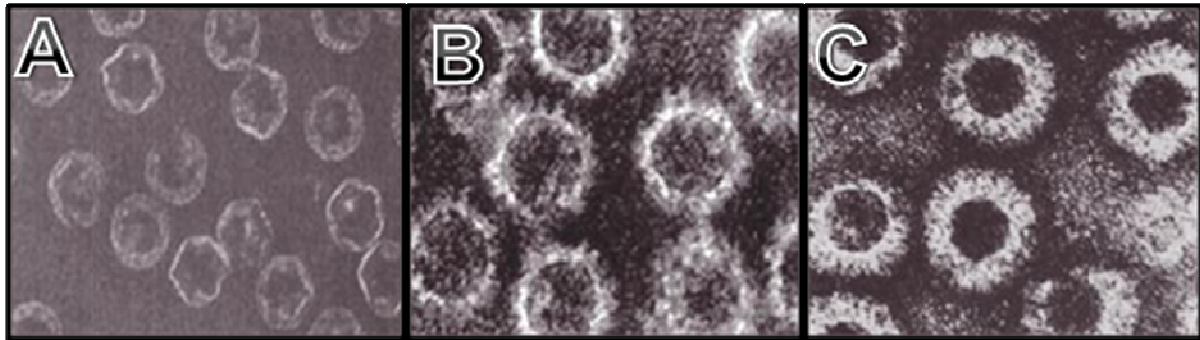


Figure 3. Electron micrograph of the concentric proteins layers of the BTV particle. Panel A shows the innermost subcore protein VP3. This layer serves as a scaffold for the deposition of the protein VP7 forming a double layered core shown in Panel B (VP3 + VP7). The spiky appearance of the core representative of the VP7 trimers and finally Panel C showing the roughened appearance of negatively stained BTV virion with outer capsid proteins VP2 and VP5 arranged around core (Roy, Boyce et al. 2009).

3.1. BTV structural organization

3.1.1. Structural organization of BTV outer capsid proteins VP2 and VP5

BTV morphology was studied at high resolution using cryo-electron microscopy and X-ray crystallography to reveal an icosahedron at a triangulation number of 13 ($T = 13$) (Grimes, Jakana et al. 1997). Closer examination of the cryo-electron micrographs revealed 60 triskelion structures which make up each virion. These structures are formed by 180 copies of 'sail-shaped' VP2 molecules (Figure 4). The tip of each VP2 trimer extends the body of the viral particle by 3 nm due to its perpendicular orientation to the plane of the virus. The base of the VP2 trimers interacts closely with the underlying core proteins (Roy 1996; Mertens, Diprose et al. 2004; Roy 2008). To date, there is no VP2 crystal structure available due to the difficulty associated with large scale production of purified BTV-VP2. This in-turn means that definitively, one cannot predict the position of BTV-VP2 constituent amino acids within the proteins tertiary structure and hence the amino acids comprising the neutralizing epitopes (Meyer, personal communication). Interspersed within the VP2 trimers and more underlying are 360 copies of globular VP5 which are arranged as 120 trimers, each of which are 6 nm in diameter (Figure 4). The upper regions of VP5 are not covered by VP2 and the tails of these globular structures are in contact with the side of the viral inner core forming channels (Roy 1996; Mertens, Diprose et al. 2000; Nason, Rothagel et al. 2004; Roy 2008).

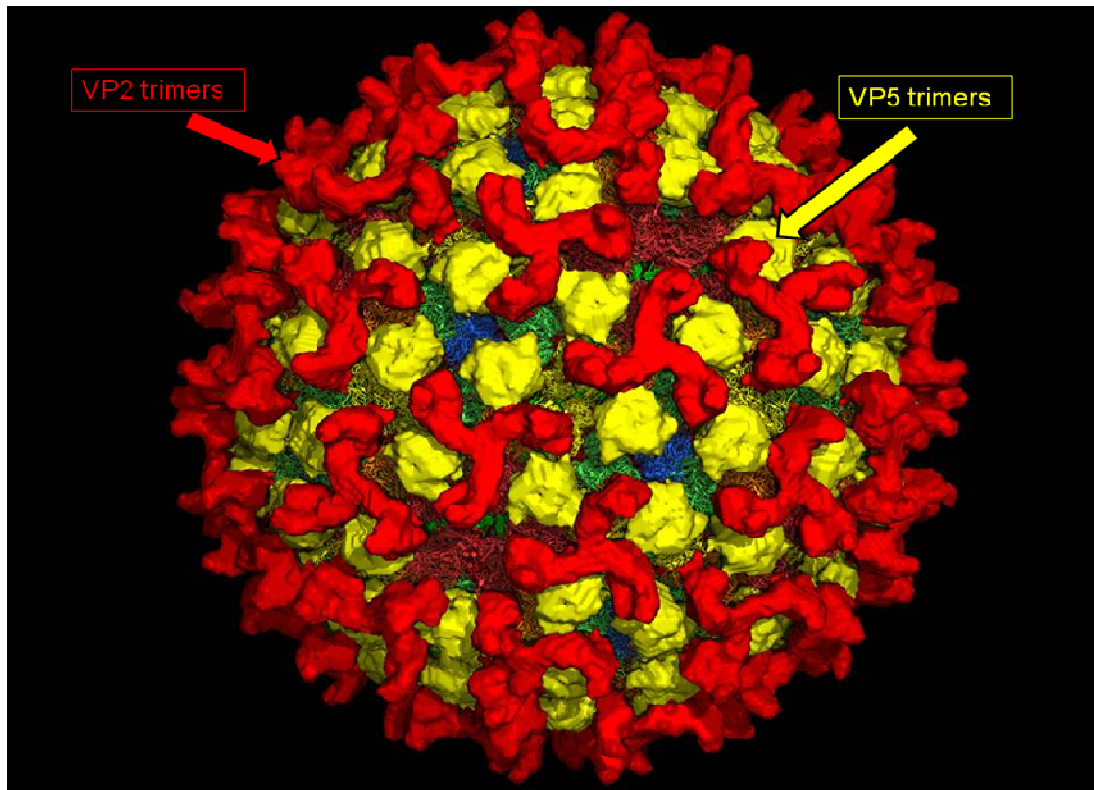


Figure 4. Surface representation of cryo-electron micrograph of a BTV particle. Each virion is surrounded by 60 trimers of outer, most exposed capsid protein, VP2 (red). Interspersed within the VP2 are 120 trimers of a globular protein, VP5 (yellow) (Roy 1996).

3.1.2. Structural organization of BTV core and associated proteins

The core of BTV also displays an icosahedral symmetry of diameter 73 nm and has a triangulation number of 13 ($T = 13$). The surface of each core is made up of 260 VP7 trimers i.e. 780 VP7 molecules per virion. Each trimer forms knob-like protrusions which take up varying positions at the surface of the core. These trimers are identified with increasing distance from the 5-fold axes (P, Q, R, S) and T at the 3-fold axes. The trimers are arranged around 132 distinct channels as six-membered rings (five-membered rings are found at the vertices of the core) (Prasad, Yamaguchi et al. 1992; Grimes, Burroughs et al. 1998). Each trimer with an upper and lower domain, are orientated such that the upper anti-parallel β -sandwich domain of a monomer rest on the lower α -helical domain of its neighboring monomer (Grimes, Basak et al. 1995). It is speculated that the channels (18 nm wide and 8 nm deep) which are associated with the tails of the VP5 trimers may allow for the transportation of metabolites to the transcriptional sites and nascent mRNA out of the core into the host cytoplasm for viral protein synthesis (Roy 1992). The lower domain of VP7 interacts with the inner most capsid shell of the viral core, the subcore. The subcore is composed of 120 copies of VP3 molecules which are arranged as 60 dimers with a $T = 2$ icosahedral pseudo-

symmetry. In order for a complete capsid shell, the VP3 must take up 2 different conformations namely A or B. These chemically identical molecules undergo conformational shifts between their three distinct apical, carapace and dimerisation domains thus facilitating the closing of the surface of the icosahedron (Mertens and Diprose 2004).

Due to the close association with the viral RNA, the exact location of the minor proteins i.e. VP1, VP4 and VP6 could not be established (Grimes, Burroughs et al. 1998). However cryo-electron analysis of recombinant core-like particles exhibited a flower-like density found below the 5-fold axes of the icosahedron and attached to the under surface of the VP3 layer. Based on previous reovirus research, it was concluded that the central mass is probably VP1 while the density projecting sideward is VP4. VP6 is believed to be located directly below the VP3 layer. Although these proteins position is speculation based on a related virus, it seems to be consistent with the proposed BTV genome organization suggested by X-ray crystallography (Zhang, Walker et al. 2003). Electron density maps of the BTV particle revealed electron dense areas within the sub-core which are not consistent with viral proteins. Experiments conducted by Grimes et al. (1998) and Gouet, et al. (1999) modeled the total RNA genome in four concentric layers thereby allowing for the organization of the 10 double stranded RNA segments ranging from 822 to 3954 base pairs in a limited space within the BTV core (Grimes, Burroughs et al. 1998; Gouet, Diprose et al. 1999).

3.2. BTV molecular biology

BTV has ten discrete double stranded RNA genome segments designated Large/L1-3, Medium/M4-6 and Small/S7-10. Each segment except S10 contains a single open reading frame which encodes each of the viral polypeptides. The RNA genome segment S10 possesses two open encoding NS3 and NS3A. The ten double stranded RNA genome segments encodes four structural proteins (VP2, 3, 5 and 7), three minor proteins (VP1, 4 and 6) and four non-structural proteins (NS1, 2, 3 and 3A) within in infected cells (Table 1) (Mertens, Brown et al. 1984; Roy 1992).

Four non-structural proteins have been identified in BTV infected cells. These NS proteins share a high degree of sequence conservation (96 %) among the BTV serotypes (Roy, Marshall et al. 1990). NS1 and NS2 are synthesized in abundance and are associated with viral tubules and VIBs respectively. The viral tubules which are attached to the filament component of the cell cytoskeleton are made up of NS1 only. The presence of many such tubules in a virally infected cell is therefore the multimeric form of the protein (Urakawa and Roy 1988). The NS1 is a cysteine rich protein and with several hydrophobic domains which is believed to be involved in the tubule formation.

Table 1. BTV constituent protein coding assignments (Roy 1992).

RNA segment	Protein & MW	Location	Function
L1	VP1 (150 kDa)	Subcore	RNA polymerase
L2	VP2(110 kDa)	Outercapsid	Serotype determinant, Structural
L3	VP3 (103 kDa)	Subcore	Scaffold for VP7, Structural
M4	VP4 (78 kDa)	Subcore	Capping enzyme
M5	VP5 (59 kDa)	Outercapsid	Structural
M6	NS1 (64 kDa)	Nonstructural	Tubules???
S7	VP7 (38 kDa)	Core surface	Group specific, Structural
S8	NS2 (41 kDa)	Nonstructural	Viral inclusion bodies
S9	VP6 (76 kDa)	Subcore	elicase
S10	NS3 (25 kDa) NS3A (24 kDa)	Nonstructural	Involved in virus release

The cysteine residues are conserved among the BTV serotypes. Cryo-electron microscopy revealed that the NS1 protein occupies a dimeric conformation arranged as helically coiled ribbons of 1000 nm in length (Marshall, Fayard et al. 1990; Roy 1992). NS2 is a phosphorylated protein rich in charged amino acids. In vitro expression of NS2 results in the synthesis of VIB-like structures similar to that found in virally infected cells (Huisman and Els 1979). The protein shows a strong affinity for single stranded RNA. Studies involving truncations of the protein showed that the N-terminal domain was responsible for this interaction and further dephosphorylation of NS2 did not affects it RNA binding capacity (Thomas, Booth et al. 1990; Roy 1992). In contrast to the highly expressed NS1 and NS2 in infected cells, NS3 and NS3A are synthesized in relatively small amounts (Mertens, Brown et al. 1984). These proteins possess two hydrophobic domains which are believed to serve as transmembrane domains and two glycosylation sites. Recombinant vaccinia virus expressing NS3 and NS3A revealed a close relationship between these proteins, intracellular

vesicles and the plasma membrane. This relationship suggests that these proteins may play a role in the release of the BTV progeny virus from infected cells (Hyatt, Gould et al. 1991; Roy 1992).

VP1 is the largest BTV protein present at approximately six molecules per virion (Huisman and Van Dijk 1990). The proteins size, location and amino acid sequence suggests it functions as the viral RNA polymerase. Sequence analysis of VP1 confirmed the presence of GDD motif which is characteristic of a RNA polymerase. The VP1 sequence also shows a strong homology to existing viral RNA/DNA polymerases and subunits of eukaryotic and prokaryotic polymerases (Roy, Fukusho et al. 1988). The BTV mRNA is capped and methylated at the 5' end during transcription. The RNA capping activity is carried by enzyme guanylyl transferase in reovirus and rotavirus (Mao and Joklik 1991; Pizarro, Sandino et al. 1991). Experiments conducted by Le Blois et al. in 1992 involving recombinant baculovirus expression of VP4 in Sf cells showed a strong affinity between VP4 and GTP. This suggests that VP4 is in fact the guanylyl transferase of BTV and is involved in catalyzing mRNA capping (Le Blois, French et al. 1992). VP6 is the third minor core protein with its N and C terminal domain separated by a mid glycine rich region. The protein displays a high affinity for single stranded and double stranded RNA, an interaction believed to be independent of its tertiary structure since it maintained this ability post SDS treatment (Roy, Adachi et al. 1990). Sequence analysis revealed a characteristic helicase motif and thus it is believed that VP6 functions as the viral helicase which unwind the duplex RNA prior to cRNA synthesis (Roy, Adachi et al. 1990)

The BTV core is composed of two structural proteins VP3 and VP7. VP7, found on the outer surface of the core, is highly hydrophobic. All BTV serotypes contain one conserved lysine residue with VP7 which suggests a role in structure (Kowalik and Li 1991). VP7 is naturally soluble during *in vitro* synthesis using baculovirus expression system. This has allowed for a more or less complete characterization of the protein including its crystal structure (Basak, Stuart et al. 1992). The VP7 forms trimers which stabilize the subcore layer made up of the hydrophobic VP3. VP3 in turn is responsible for the structural integrity of the viral core (Roy 1992).

3.2.1. Molecular biology of BTV outer capsid proteins VP2 and VP5

BTV core is surrounded by outer capsid proteins VP2 and VP5. Due to its locality, VP2 is responsible for receptor binding thereby initiating viral entry into the host cell. *In vitro* expressed VP2 has a strong affinity for glycoprotein A (GPA), the sialoglycoprotein component of erythrocytes. This interaction allows for the attachment of BTV to susceptible cells causing hemagglutination (Hassan and Roy 1999). Logically it stands that preventing such an interaction will cause a inhibition of viral infection. At this point there are two vaccine candidates, the ligand, VP2 or the receptor, GPA. GPA, a 36 kDa protein is well characterized however its functional importance remains poorly understood (Hassoun, Hanada et al. 1998). It is hypothesized that GPA facilitates the expression,

post-translation modification and translocation of band 3 anion transporter to the membrane of red blood cells (Bruce, Groves et al. 1994; Hassoun, Hanada et al. 1998). Band 3 is responsible for the HCO_3 efflux of from the red blood cell in exchange for Cl^- (Jennings 1989; Groves and Tanner 1992). Whatever the function may be, it is certain the GPA is not an obsolete protein present to serve as an attachment site for the entry of viruses such as BTV and Sendai virus and parasites such as *Plasmodium falciparum* (Perkins 1981; Wybenga, Epand et al. 1996; Hassan and Roy 1999). Also it is unclear whether glycophorin A is the only receptor responsible for BTV entry (Hassan and Roy 1999). It therefore becomes apparent that an attractive vaccine candidate would be BTV outer capsid protein VP2.

Within host cells, VP2 interacts with vimentin which not only regulates the sub-cellular localization of VP2 but also is responsible for the association between mature BTV particles and intermediate filaments which contributes to virus egress (Hassan and Roy 1999; Bhattacharya, Noad et al. 2007). VP2 is the most variable and thus the serotype determinant of BTV. VP2 amino acid sequence comparison of all BTV serotypes revealed perfect correlation between the variations in sequence. More than 80% of BTV isolates showed a 48 – 64% variation between the serotypes with highly variable regions lying between amino acid 590 - 690 and 150 – 290 (Huismans, van Staden et al. 2004). Despite the sequence variability of VP2, the proteins charge distribution, hydrophobicity and position of certain cysteine residues appear to be conserved across the serotypes (Maan, Maan et al. 2007). VP2 also contains serotypic and to a lesser extent serogroup neutralizing epitopes (discussed in BTV subunit vaccination strategy below). These conformational dependent epitopes have been localized to the N-terminal region of the protein (DeMaula, Bonneau et al. 2000).

VP5 is the second most variable protein. In its monomeric conformation, it is divided into an amino terminal coiled domain which is able to form 2 amphipathic helices, a mid-flexible hinge region and a globular carboxy terminal domain. The amphipathic helices are a uniquely conserved feature of VP5 which plays an important role in membrane destabilization (Hassan, Wirblich et al. 2001; Forzan, Wirblich et al. 2004; Roy 2008). Previous studies have shown that despite the location of VP5, antibodies raised against the purified protein failed to neutralize the virus (Huismans, van der Walt et al. 1987; Marshall and Roy 1990). It is for this reason that BTV-VP5 was not considered as a vaccine candidate in the current study.

3.3. Current BT vaccination strategy

The current vaccination protocol against BT entails the use of live-attenuated vaccines manufactured by Onderstepoort Biological Products in South Africa. The three Onderstepoort Bluetongue Vaccines are prepared in cell culture and freeze-dried. The first bottled preparation contains

attenuated BTV serotypes 1, 4, 6, 12 and 14; the second bottled preparation contains attenuated BTV serotypes 3, 8, 9, 10 and 11 while the third bottled preparation contains attenuated BTV serotypes 2, 5, 7, 13 and 19. The three vaccines are administered individually at intervals of three weeks and are certified for use in sheep only. The efficacy of the vaccines in other ruminants has not been established. The success of BTV live-attenuated vaccine is due to the high immunogenicity associated with the least number of immunizations. This is possibly due to the presentation of relevant epitopes in their cognate native form. Secondly, the entire BTV particle may elicit a stronger immune response due to the exposure of a more epitopes. Finally, the vaccine maintains replicative capacity which continuously rouses the immune system (OIE Manual of Standards for Diagnostic Tests and Vaccines 1996; Russo, Turin et al. 1997; Lubroth, Rweyemamu et al. 2007). Despite the cost efficiency and significant, prolonged immunity in sheep, there still safety apprehensions regarding the use of the Onderstepoort BT Vaccines due to the following (Veronesi, Hamblin et al. 2005): a) The presence of the attenuated virus in vaccinated sheep, one cannot distinguish between vaccinated and naturally infected animals i.e. lack of DIVA. Thus BT free countries prohibit sheep importation from countries with BT. This places a strain on the export industry of a country with BT and results in millions being lost every year. b) Certain countries e.g. European countries prohibit the use of the current live-attenuated vaccine. c) If vaccine transmission occurs, reversion of the attenuated strain to pathogenesis might occur. d) The presence of nucleic acids in the vaccine suggests the possibility of genetic recombination between the vaccine strain and field wild-type strains which may result in novel and possibly more virulent serotypes. e) There are reports of the BTV vaccine being teratogenic when administered to pregnant sheep and lowering fertility in rams with regard to quantity of sperm. f) Attenuated strains which generate titres of < 1000 plaques forming units (PFU) per ml of blood at the corresponding height of viraemia will be selected for vaccine purposes. It is suggested that at < 1000 PFU / ml blood at the height of viraemia will prevent transmission of the vaccine strains by the *Culicoides* vectors. This has not been established however research conducted by Venter and Paweska in 2007 showed a recovery of AHSV attenuated vaccine from the *Culicoides* vectors (OIE Manual of Standards for Diagnostic Tests and Vaccines 1996; Venter and Paweska 2007).

4. VACCINATION STRATEGIES

A vaccine constitutes an antigenic preparation which elicits an immune response when an individual is challenged with such a preparation. It serves as one of the most important available strategies to prevent infectious disease (Russo, Turin et al. 1997; Liljeqvist and Stahl 1999). The immune response includes antibody synthesis with activated T-lymphocytes and killer cell recruitment thus affording protection or lessening of the severity of vaccinated individuals against subsequent

infections of the same or similar nature. The birth of vaccinology may be traced to English doctor, Edward Jenner and his discovery of the smallpox vaccine in 1798. He found that immunization with the antigenically similar but less virulent cowpox virus offered immunity against the virulent smallpox virus (Russo, Turin et al. 1997; Shams 2005; Lombard, Pastoret et al. 2007). The concept of vaccination however, was applied well before Jenner. In AD 590, Chinese sniffed powdered smallpox pustules as an immunization strategy (Minichiello 2002). Nevertheless, Jenner's finding sparked much research and thus further understanding of the vaccine concept eventually leading to the worldwide eradication of smallpox and a dramatic reduction of diseases including diphtheria, measles, mumps, polio and tetanus (Dorner and Barrett 1999).

Clearly this is a demonstration of human vaccinology with its focus being on individuals. However the importance of veterinary vaccinology should not be ignored. Veterinary vaccinology, which started with the development of the foot and mouth disease (FMD) vaccine in the late 19th century, is applicable to companion, wild and livestock animals (Lombard, Pastoret et al. 2007). The relationship between human and veterinary vaccinology becomes apparent when considering veterinary vaccinology may also protect humans from diseases common to both populations. The distinction between the two 'medicines' lie in the flexibility of ethical issues surrounding veterinary vaccinology. Such issues include initial experimentation on animals to priority of trade and industry rather than the welfare of animals. Mass culling of infected or potentially infected animals is often carried forth instead of the initial treatment (Lombard, Pastoret et al. 2007).

Veterinary vaccinology targets the improvement of animal health sector through a) cost efficient prevention and control of infectious diseases, b) improving animal welfare and c) a corresponding decrease of in the production cost of food animals. So when considering the advantages of veterinary vaccinology to humans, besides the obvious of avoiding an economic loss caused by disease, vaccines may substitute and/or reduce mass consumption of varying veterinary drugs. The resultant effect of this is a reduction in the number of side effects caused by the drugs and a decrease in the amount of drug residues found in the animal products. Thus veterinary vaccinology also part takes in the enhancement of the public health (Shams 2005; Lombard, Pastoret et al. 2007).

4.1. The immune response to infection

In retrospect, the immune system is comprised of two parts i.e. the innate and adaptive/acquired immune system. The innate immune system, consisting of cells and mechanisms, protects the host in a non-specific manner and does not confer life-long immunity to the host against further challenges from the same or similar pathogen. The adaptive immune system, in contrast, consists

of highly specialized cells which provide an antigen-specific immunity thereby protecting the host against future infections from the same or similar pathogens. Although the two systems may be divided for simplicity in understanding, they are interlinked and it is the innate immune system which activates the adaptive immune system (Delves, Martin et al. 2006). A vaccine elicits an immune response by the stimulation of the host adaptive immune system. An adaptive immune response may include a humoral response (production of antibodies) and/or cell-mediated response depending on the cell type involved (Takahashi 2003).

Humoral immunity is a response toward an extracellular pathogen residing or replicating outside the host cells in the alimentary, urogenital, and respiratory tracts. Such pathogens escape phagocytosis and corresponding death by phagocytes such as monocytes, neutrophils and macrophages. Humoral immunity proceeds via B-cell recognition of foreign material followed by their development into plasma cells. These plasma cells divide and differentiate thereby producing various classes of antibodies. A humoral response may also be a common occurrence against intracellular pathogens such as viruses, certain bacteria and parasites since infection by these pathogens begin with the initial adherence to host cells (Shams 2005). The opsonization of these parasites by antibodies increases their susceptibility to internalization and degradation by phagocytes bearing a corresponding Fc receptor. This in-turn may inhibit their adherence and subsequent entry into host cells ergo preventing infection and disease. While the humoral response may allow for the recognition of foreign material, it depends on the cell-mediated immunity i.e. T-cells for its (B-cell) division and differentiation in order to produce antibodies (Minichiello 2002; Delves, Martin et al. 2006).

T-cells are lymphocytes which constitute cell-mediated immunity. Several T-cells are recognized. Pathogens, once internalized by specialized phagocytic cells such as B-cells, dendritic cells, Langerhan cells and macrophages (known as antigen presenting cells (APCs) evade humoral immunity. Cell mediated immunity is therefore relied upon for the restriction and removal of such pathogens (Kaufmann, Fensterle et al. 1999). Pathogens taken up by APCs are processed into smaller peptides and presented via major histocompatibility complex class II (MHC class II) while antigens from other nucleated cells are processed and presented via MHC class I on the surface of the cells (Delves, Martin et al. 2006). In general MHC class II activates or re-activates helper T-cells (T_h) cells expressing surface protein CD4. CD4+ T-cells allow for the induction i.e. recognition and subsequent T-cell activation of the immune response as well as the effector response i.e. cytokine and cytolytic activity. Depending on the pathogen and the response required, CD4+ T_h cells differentiate into either T_h1 or T_h2 cells (Type 1 or Type 2 helper T-cells). T_h1 cells stimulates cytotoxic T-lymphocyte (CTL) activity which kills target cells such as virally infected cells while T_h2 cells promotes proliferation and differentiation of antigen-specific B-cells as well as induce antibody

class switching (Delves, Martin et al. 2006). Since MHC class II is limited to APCs, one major shortfall of CD4+ T-cells is that an infection of other cell types goes undetected by this system. This shortfall is compensated by CD8+ T-cells which recognizes and binds MHC class I present on all nucleated host cells. CD8+ T-cells possesses CTL activity. The ubiquitous expression of MHC class I suggest that CD8+ T-cells are responsible for the inspection of the entire body filtering out cells that are intracellularly infected and do not express MHC class II. It seems though CD8+ T-cells are inadequate to afford long-term protection and an optimal robust immunity is the resultant effort of both CD4+ and CD8+ T-cells (Minichiello 2002; Vossen, Westerhout et al. 2002; Shams 2005).

4.2. Requirements of an ideal vaccine

The design of an ideal vaccine should encompass the following characteristics: a) display high immunogenicity in all individuals with the capacity to elicit long-term protection b) have minimal to no side effects, c) should be effective with the first or few administrations, d) should be directed against multiple serotypes, e) should allow for the differentiation between infected and vaccinated animals (DIVA) f) the vaccine must be thermally stable and maintain its potency under different environmental conditions thus negating the requirement for expensive storage and delivery instruments, g) must be practical in terms of delivery system and usage therefore circumventing the need for specialized training in handling and administration of the vaccine and finally h) the vaccine should be manufactured in a cost efficient manner (Dorner and Barrett 1999; Savini, MacLachlan et al. 2008).

4.3. Types of vaccines

Since the smallpox vaccine, much research has been conducted in the field of vaccinology. This has led to the development of various vaccination types each with the aim of stimulating optimal immune response (Minichiello 2002).

4.3.1. Live-attenuated viral vaccines

The use of live-attenuated vaccines has been regarded as the most successful vaccine strategy to-date. The success is due to the least number of administrations (usually 1-2) which elicits a strong humoral and cellular immunity with long-term protection (Veronesi, Hamblin et al. 2005). Live-attenuated vaccines consist of whole microorganism preparations that are almost if not completely devoid of pathogenicity however still maintains its immunogenicity and replicative ability in the host. This in turn allows for the continuous stimulation of the immune system resulting in prolonged immunity (Russo, Turin et al. 1997; Shams 2005). Several approaches have been undertaken in order to generate these viral vaccines. These include:

- i. Passage of the virus in an unnatural host or tissue culture until a point is reached where viral immunogenicity is maintained but virulence is lost. The attenuated Sabin oral polio-type 1 vaccine was produced by passaging the strain in non-human primates and cell culture which resulted in 57 base substitutions, 21 of which caused a change in the amino acid sequence found throughout the genome (Dorner and Barrett 1999).
- ii. The use of a related natural pathogen from another host. The classic example of such a vaccine is Edward Jenner's cowpox vaccine against smallpox (Lombard, Pastoret et al. 2007).
- iii. The use of naturally attenuated strains as vaccines. This includes poliovirus 2 and some strains of rotavirus which were avirulent in humans (Bishop, Barnes et al. 1983).
- iv. The use of recombinant technology aimed at the removal of gene sequences contributing toward virulence thusly reducing pathogenicity (Shams 2005).

Although live-attenuated vaccines fulfill many requirements for an ideal vaccine, this strategy is not without its drawbacks. The potential reversion to virulence by spontaneous genomic mutation or genetic recombination with other wildtype strains cannot be neglected. The latter may result in potentially new pathogenic strains. This is particularly dangerous and is not uncommon if improper cell culturing techniques are utilized resulting in contaminating viruses in cultured cells. The replicative ability of live-attenuated vaccines may also have a negative influence on immunocompromised hosts (Russo, Turin et al. 1997; Plotkin 2009). The presence of active nucleic acids necessary for the replicative ability of this vaccine may also be detrimental to the host. This may be seen in retrovirus genome integration with the host genome resulting in subsequent infections. Alternatively retroviral genomes may interfere with genes coding for regulation of cell-cycle thus leading to uncontrolled cell proliferation (Plotkin 2009). The presence of all viral proteins and the viral genome means that DIVA is a challenge. This, impacts negatively on trade and industry (Greiser-Wilke and Moennig 2004).

4.3.2. Inactivated/killed whole virus vaccines

Inactivated/killed whole virus vaccines are merely intact viral preparations with an impaired replicative function. Inactivated vaccines possess all antigenic determinants however due to the absence of replicative ability, the vaccine is unable to continuously rouse the immune system and provide long-term protection. An individual therefore needs to be challenged with frequent doses of the vaccine in order to gain an equivalent protection as with live-attenuated vaccines thus making this strategy more expensive (Liljeqvist and Stahl 1999; Stauffer, El-Bacha et al. 2006). Alternatively an individual may be challenged with a larger dose of the inactivated vaccine which may result in

strong, long-term protection. However this may increase the risk of hypertension in the host (Dorner and Barrett 1999). Inactivated vaccine preparation is a highly sensitive procedure involving formaldehyde, beta-propiolactone or heat. Negligence in the vaccine production may lead to incomplete inactivation which can cause infection prior to the establishment of host immunity as in the case of an outbreak of foot and mouth disease in the United Kingdom in 1981 (King, Underwood et al. 1981; Russo, Turin et al. 1997). However, the reverse i.e. too strong inactivation is also possible. This may lead to the distortion of antigenic determinants which elicits a very poor or no immune response when administered to individuals (Russo, Turin et al. 1997). Perhaps the upside to this vaccine strategy is the inability to revert to pathogenesis or to recombine with wildtype virus and DIVA associated with inactivated vaccines. This type of vaccine may consequently provide a compromise between safety and efficacy (Russo, Turin et al. 1997).

4.3.3. Vectored vaccines/Delivery systems

Vectored vaccines allows for an immune response against different pathogens due to its design in which immunogenic regions of several pathogens are incorporated. The many advantages associated with such a vaccine strategy include, little or no side effects, stability, ease of administration and in some instances, the absence of an adjuvant (Shams 2005). Vectored vaccines include:

i. Live-vectored vaccines

This vaccine strategy involves the insertion of genes encoding immunogenic regions of different pathogens into the genome of an attenuated viral or bacterial strain. The recombinant attenuated virus or bacteria is then delivered as the vaccine. Thus an immune response is induced against the carrier attenuated strain as well as the inserted antigens. This vaccine type is largely successful due to the replicative ability of the vector and thus inserted antigen (Shams 2005; Souza, Haut et al. 2005). *Mycobacterium bovis*, Bacille Calmette Guerin (BCG) is the most successful live vectored vaccine used for the past four decades. This is due to the single inoculation required to elicit a strong CTL and mucosal immunity for prolonged periods of time with minimal side effects (Stover, de la Cruz et al. 1991; Behr 2002). Poxvirus such as canarypox and vaccinia virus is commonly used vectored vaccines which allow for high expression levels of foreign antigens. These expression vectors may form an inexpensive, DIVA vaccination strategy which allows for the inclusion of multiple serotypes. The maintenance of replicative ability of the vector implies a continuous stimulation of the immune system (Moss 1991; Russo, Turin et al. 1997). The application of live vectored vaccines in BTV research has been hailed as a success since sheep challenged with vaccinia virus and canarypox virus co-expressing outer capsid proteins VP2 and VP5 developed high titers of neutralizing antibodies and were resistant to homologous virus challenge. The *raison d'être*

behind the replacement of the current vaccine with poxvirus vaccine is very sketchy and references justify it as not fully characterized (Lobato, Coupard et al. 1997; Perrin, Albina et al. 2007). The possibility of host infection in immunocompromised individuals (Russo, Turin et al. 1997), dominant immunity toward the vector itself, tumor development in experimental trials as well as modifications of the recombinant virus in host cells which often lead to impaired replicative ability host restriction (Bender, Rowe et al. 1996; Souza, Haut et al. 2005), however, cannot be ignored. Another important disadvantage of this vaccination strategy is perhaps the possibility of an over dosage of antigen in the immunized individual. Poxvirus derived vectors are infamous for high level antigen expression in the host. However the lack of a stringent control mechanism for its replication within the host means high levels of antigen production which may lead to a corresponding hypersensitivity within the host (Bender, Rowe et al. 1996).

ii. DNA vectors

DNA vectors have been a recent and fascinating development in vaccine research. It involves the direct immunization of 'naked' DNA encoding specific antigens directly into the host cells thereby allowing expression of such antigens within the host. The expressed antigens are processed and presented via MHC class I and II molecules which elicits a CD4 and CD8 T-cell response (Dorner and Barrett 1999). Recently, the GeoVax-2 component clade B HIV-1 vaccine trial was hailed as a success in Thailand. The vaccine consists of a recombinant DNA component and a modified vaccinia Ankara (MVA) component. Both components express Gag, Pol and Env of HIV-1 virus resulting in non-infectious virus-like particles (VLPs) which elicits both a cellular and humoral response. (2009); GeoVax Press Release, 2009). DNA vaccines are administered via syringe or gene guns which deliver DNA coated beads into the host cells (Shams 2005). Albeit DNA vaccines may non-infectious as well as stable (eliminating specialized cold-chain requirement), extraneous DNA in a foreign cell could pose various disadvantages. Integration of the vaccine with the host chromosome is possible which could interfere with genes involved in regulating cell cycling. This might lead to uncontrolled cell proliferation (Shams 2005). The production of anti-DNA antibodies against the foreign DNA is also a concern since such antibodies could be detrimental toward host DNA due to common chemical characteristics associated with DNA molecules (Dorner and Barrett 1999). Also it is not yet possible to administer DNA vaccines to mucosal surfaces although research is been conducted (Shaw, Gaerthe et al. 2000).

iii. Plant vectors/vaccines

Plant vaccines allow for the expression of antigens in their native conformations within edible tissue. This means that the vaccine itself is edible. This eliminates the need administration via for trained

staff, specialized storage facilities and instruments associated with vaccine delivery i.e. syringes and needles. The downside to such an innovation however, includes a) enhancing efficacy associated with antigens which are administered orally, b) the stability of antigens in combination with digestive enzymes and exact dosage required to elicit a significant immune response (Shams 2005). Despite these drawbacks, there has been much success with transgenic plant expression of rabies glycoprotein and *E. coli* heat labile enterotoxin (McGarvey, Hammond et al. 1995; Mason, Haq et al. 1998).

iv. Display protein vectors

The insertion of DNA sequences encoding an immunogenic region of a protein into immunogenic and immunologically exposed regions of a second protein may result in an immune response directed toward the inserted peptide. This is principle technology behind display vectors where the second peptide acts as a display system for the presentation of the inserted segment to the immune system (Georgiou, Stathopoulos et al. 1997). Unlike live-vectored vaccines or DNA vectored vaccines, the display system with the inserted peptide sequence is expressed prior to its immunization into the host. This means that a homogenous antigen preparation is administered to the host eliminating the possible shortfalls associated with live-vectored and DNA vaccines (Liljeqvist and Stahl 1999). The success of such a strategy may be seen in the case of AHSV and BTV VP7 display vectors. These vectors have been engineered to allow for the insertion of foreign peptides (up to 240 amino acids) at different hydrophilic sites on the top exposed domain of the VP7 trimer (Henk Huismans, personal communication).

4.3.4. Subunit vaccines

Subunit vaccinations involve the use of distinct formulations containing single or mixed immunogenic proteins (subunits) of single or various pathogens. The underlying basis of such a strategy relies on the identification and selection of the immunogenic regions of the pathogen(s), the subsequent cloning of such regions into an expression vector, followed by the expression of the antigen in foreign host systems such as bacterial, insect, mammalian or yeast cells. The expression product in a soluble form is easily purified and serves as the vaccine (Koths 1995; Liljeqvist and Stahl 1999; Shams 2005). Subunit vaccines are advantageous over live-attenuated vaccinations due to the lack of nucleic acids and toxic components (discussed under live-attenuated vaccines above). The expression of “subunits” of a pathogen(s) allows for the retrieval of pure antigen, free of extraneous material which usually accompanies heat or chemically inactivated vaccines. The antigen is expressed at relatively high levels, at corresponding low costs (Russo, Turin et al. 1997; Liljeqvist and Stahl 1999).

The first successful application of a subunit vaccination strategy involved the expression of Hepatitis B surface antigens in mammalian and yeast cultures, largely replacing the plasma-derived vaccine. Initially, Hepatitis B antigens were expressed in bacterial systems. This was unsuccessful since the complex protein structure and the lack of post-translational modification machinery in bacteria resulted in aggregates of the proteins (Valenzuela, Medina et al. 1982; Dorner and Barrett 1999). However in 2008, Song, et al. developed an efficacious recombinant influenza vaccine in a bacterial system. The fusion protein linking the globular head domain of the hemagglutinin antigen with toll-like receptor-5, flagellin, elicited a strong humoral response which protected the mice during a lethal challenge with the virus (Song, Nakaar et al. 2008). Also in 2008, Li, et al. demonstrated the protective capacity of baculovirus expressed foot-and-mouth disease virus (FMDV) capsid proteins. The protease encoding regions P1-2A and 3C Asian FMDV 1/HNK/CHA/05, expressed in silkworm larvae, was used as a subunit vaccine in cattle. The vaccine elicited complete protection against a homologous virulent virus challenge in four of the five animals with assuage of the clinical symptoms (Li, Yi et al. 2008).

In vitro synthesis of an antigen often leads to large amounts of insoluble proteins which do not resemble their native-like structure. This is problematic since vaccination strategies call for high level production of soluble protein. The degree of complexity of a given protein e.g. in terms of the number of disulphide bonds present, a large number of hydrophobic regions which could potentially cause misfolding which may lead to aggregation of proteins, etc. would therefore determine its propensity to express in a non-native and aggregated state (Georgiou and Valax 1996; Idicula-Thomas and Balaji 2005). This problem may be elevated if the position of the neutralizing epitopes were known. Truncated versions of the protein comprising the essential immunogenic regions and fewer hydrophobic domains, glycosylation sites and cysteine residues may be synthesized *in vitro* and used as a potential subunit vaccine (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009). In 2004, Kazunari and Arakawa expressed a 180 amino acid N-terminal truncation of the S1 subunit of *Bordetella pertussis* in an *E. coli* system and demonstrated the immunogenic efficacy in mice (Kamachi and Arakawa 2004). In 2009, Bonafe et al. showed that a truncated West Nile virus envelope protein (rWNV-E) produced in baculovirus infected insect cell line may be used as a potential vaccine candidate. The rWNV-E antigen which was stable for approximately 8 months at 4 °C, induced virus neutralizing antibodies in mouse and hamster WNV challenge models. The antigen also induced a humoral response in naïve foals and tested safe with regard to side effects when injected at high doses in rats. The study concluded that a baculovirus expressed truncation of WNV may be safe and stable vaccine candidate for protection against the virus (Bonafe, Rininger et al. 2009). Liu et al. previously reported difficulty in the expression of full-length A1 subunit of shiga toxin 2. However late 2009, Liu and associated

reported the expression of a truncated version of the protein in which 15 amino acids from the carboxyl terminal which display strong hydrophobicity and low antigenicity were deleted. Antibodies raised against the truncation were able to neutralize the shiga toxin 2 *in vivo* and *in vitro* thus implicating the truncation as a potential subunit vaccine candidate (Liu, Zeng et al. 2009). A truncated version of *Pasteurella multocida* toxin (PMT) was expressed in a bacterial system and used as a subunit vaccine against atrophic rhinitis in pigs. Subsequent to challenge, the immunized pigs developed no or mild atrophic rhinitis suggesting the vaccine potential of the truncated PMT (Seo, Pyo et al. 2009).

Often subunit vaccines are too “small” and therefore far less reactogenic than is required of a vaccine. The subunit or hapten (as they are referred to) may however be coupled to a T-cell dependent carrier protein or an adjuvant. An immune response against the carrier protein in essence increases the response against the hapten. Another disadvantage of a subunit vaccine is that the immune response may be weaker in comparison to the response against the entire pathogen (Dorner and Barrett 1999). Subunit vaccinations may be extended to three other related vaccine types i.e. peptide-based vaccines, anti-idiotypic vaccines and virus-like particles.

I. Peptide-based vaccines

Peptide-based vaccines constitute the use of linear peptide sequences containing B- and T-cell epitopes as a vaccine strategy. The relevant immunogenic epitopes of a pathogen (usually B-cell epitopes) are coupled to a carrier protein (a source of T-cell epitopes) and presented to the immune system. Since this strategy employs the use of selected peptide sequences, it eliminates the toxic effects associated with other polypeptide sequences present on a pathogen (Chai, Clavijo et al. 1992; Ben-Yedidia and Arnon 1997). Currently malarial and HIV vaccine research involves the use of multiple antigen peptide system or MAPS. This combines B-and T-cell epitopes with a polylysine core thus negating the requirement of a carrier. The necessity of a quantifiable homogenous amount of antigen in MAPS consents to high levels of antibody specificity (Chai, Clavijo et al. 1992; Dorner and Barrett 1999).

II. Virus-like particles

Possible shortcomings to a subunit vaccine approach are a) the avidity of an immune response against an entire infectious agent might be greater than the immune response toward individual components due to the presence of significantly greater number of exposed epitopes on the entire pathogen and b) isolated expression of an immunogenic protein may result in the loss of the complex native conformation of such a protein as found on the pathogen itself (Noad and Roy 2003). Such shortcomings may be addressed by virus-like particles (VLPs). VLPs arise from the auto-

aggregation property of some eukaryotic and viral proteins when they are concurrently expressed in the same expression system (Russo, Turin et al. 1997). The presence of the different constituent proteins of an agent in effect means each protein is subjected to the same molecular and chemical forces as in the pathogen itself. This allows for a “more” native presentation of the protein subunits to the immune system. VLPs also accommodates for the difficulty associated with locating the exact neutralizing epitopes by presenting a range of epitopes. The absence of nucleic acids also makes this strategy particularly attractive since there is no likelihood of recombination with wild-type and/or reversion to pathogenesis (Russo, Turin et al. 1997; Noad and Roy 2003). Currently the most successful commercially available VLP vaccine is Gardasil and Cervarix which protects against human papillomavirus (HPV) vaccine 16 and 18 respectively. The vaccine is based on the inherent ability of the major capsid protein L1 of HPV to self assemble into a VLP which takes up native viral structure and immunogenicity (Villa, Costa et al. 2005; Harper, Franco et al. 2006; Schellenbacher, Roden et al. 2009). The downside of VLP vaccine strategy however lies with the difficulty in large scale production, the decrease in stability of the VLPs over time and specialized cold-chain requirement for storage (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). Large scale production of VLPs results in products that are not necessarily consistent in terms of composition and architecture (Pattenden, Middelberg et al. 2005). This downside taken in account with the effectiveness of the HPV vaccine and the amount of money the vaccine will save the health care system explains the reasoning behind the high cost associated with the HPV vaccine (around \$360.00 ~ R2880.00).

4.4. Previous BTV-VP2 subunit vaccination strategies

Subunit vaccines play an important and extensive role in BTV vaccine research. Previous studies into a BTV-VP2 subunit vaccine strategy involved BTV-VP2 only, VP2 in combination with VP5 and VLPs (VP2 + VP3 + VP5 + VP7) from one serotype or a cocktail of serotypes (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). The most elegant vaccination design due to its simplicity was the utilization of the outercapsid protein VP2 by itself. In 1981, Huismans and Erasmus showed a correlation between the BTV-VP2 precipitating antibodies isolated from BTV-infected sheep and neutralizing antibody titres (Huismans and Erasmus 1981). Later in 1987, Huismans, et al. demonstrated BTV neutralization in sheep using isolated BTV-VP2. The VP2 was stripped of authentic BTV particles by treatment with a monovalent or divalent salt buffer at a low pH combined with a Freon extraction method. The isolated VP2 was soluble due to its removal from authentic BTV. The sheep which were inoculated with soluble BTV-VP2 elicited virus neutralizing antibodies and were resistant to a homologous viral challenge (Huismans, van der Walt et al. 1987). Naturally, a resistance to a homologous challenge

is expected since VP2 is serotype determinant of BTV (Roy 1992). The yield of the isolated VP2 was however dependent on the virus stock and the solubility of this antigen decreased upon long-term storage (Henk Huismans, personal communication). The use of Freon is also now considered unsuitable since it has been implicated in the ozone layer depletion and further rising environmental concerns (Taylor 1996). Huismans, et al were also the first to demonstrate that the neutralizing epitopes of BTV-VP2 are conformational dependent since BTV-VP2 that was purified from polyacrylamide gels failed to induce neutralizing antibodies (Huismans, van der Walt et al. 1987).

Also in 1987, Inumaru and Roy demonstrated BTV neutralizing activity with antibodies raised against BTV(10)-VP2 in mice and rabbits. However this study entailed an *in vitro* synthesis of BTV-VP2 in Sf cells using baculovirus expression system (Inumaru and Roy 1987). In 1990, Roy, et al. reported complete serotypic protection in sheep using unpurified BTV(10)-VP2 as an antigen which was administered at >100 µg/dose or equivalent. Interestingly, to a lesser extent the antisera neutralized BTV serotypes 4, 11, 17 and 20. Further studies involving antisera raised against BTV-VP2 of serotype 1, 2, 11, 13 and 17 showed homologous virus neutralization as well as a small degree of cross-protection. The protection afforded by *in vitro* synthesized BTV-VP2 is noteworthy since the antibodies raised against BTV-VP2 alone recognized and neutralized the virus, it suggests that individually expressed BTV-VP2 takes up a conformation similar to BTV-VP2 found on the authentic bluetongue particle and therefore exposes the appropriate epitopes in the native conformation to the immune system (Roy, Urakawa et al. 1990).

Challenge with less of the same protein i.e. ca. 50 µg of VP2 administered in combination of ca. 20 µg VP5 offered complete protection against homologous virulent BTV challenge and elicited higher titers of neutralizing antibodies. The reasoning behind the significant increase in neutralization and protective response is unknown (Roy, Urakawa et al. 1990). However it is believed that VP5 boost the immune response indirectly through its interaction with VP2. The VP5 probably enhances the VP2 conformation which then displays a more native state as found in intact BTV particles. It should be noted that purified BTV-VP5 was capable of inducing VP5 precipitating antibodies that failed to neutralize BTV (Huismans, van der Walt et al. 1987; Marshall and Roy 1990; Roy, Urakawa et al. 1990). It was for this reason that BTV-VP5 was not under consideration as a vaccine candidate in the current study.

Various studies were also conducted in order to identify and characterize the neutralizing determinants of BTV-VP2. The research outputs were often conflicting with regard to the number of neutralizing epitopes present on BTV-VP2 however there is a general consensus of the location defined to the N-terminal region of the protein regardless of the virus serotype (Mecham and Jochim 1990; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; Athmaram, Bali et al. 2007). In

2007, Athmaram, et al. described the immunogenic potential of a 45 kDa truncation of BTV-VP2 in rabbits. The C-terminal truncation was expressed at high levels (284 mg/L) in *Pichia pastoris* (Athmaram, Bali et al. 2007).

Vaccination trials involving BTV VLPs also showed considerable success. VLPs, containing BTV VP2, VP3, VP5 and VP7, were administered in various doses ranging from 10 – 200 µg/sheep. The results from VLP trials revealed that a 10 µg dosage of BTV-10 VLPs afforded protection against homologous BTV challenge for a minimum of 15 months. Interestingly VP2 constituted 10-20% of the VLP mass. Finally vaccination trials involving a cocktail of VLPs containing different serotypes were evaluated. These trials revealed that a low vaccination dosage of the VLP cocktail (i.e. ca. 10 µg BTV-10 and -17) could afford protection against homologous virus challenge and an increase in dosage (ca. 50 µg) afforded cross-protection (i.e. against BTV-4). Similar results were obtained when a cocktail vaccine containing BTV-2, -10, -11, -13 and -17 protected the vaccinated sheep against BTV-16. Thus it becomes apparent that complete protection as well as cross-protection by use of the VLP cocktail vaccination is dependent on the challenge serotype and quantity of antigen (Roy 1990; Roy 1992; Roy 1992; Roy, French et al. 1992; Roy, Bishop et al. 1994; Urakawa, French et al. 1994; Lubroth, Rweyemamu et al. 2007).

In addition to the excellent immune response and the prolonged protection offered by BTV subunit vaccination strategies, they also offer the following advantages: a) the antigens can be obtained in a relatively pure form. This eliminates the possibility of adverse allergies in the host involving BTV nucleic acids or proteins from expression systems. b) The absence of nucleic acids eliminates the possibilities of transmittance by vectors, recombination with other wild-type BTV strains and the reversion to pathogenesis. c) As in the case of VLPs, each protein subunit mimics the conformation as found on the authentic BTV thereby allowing for efficient immune presentation. d) The utilization of only certain proteins in subunit vaccinations leaves room for the development of an appropriate BTV detection protocol using other proteins not included in the vaccine. This will allow for the discrimination between vaccinated and infected sheep thereby permitting importation of sheep into countries free of BTV. e) Various expression systems allow for an inexpensive yet high level production of protein subunits. f) Subunit vaccines comprising of individually expressed proteins are relatively stable which negates the requirement of expensive cold chains as required by live-attenuated vaccines during transportation (Russo, Turin et al. 1997; Dorner, Eibl et al. 1999).

Despite the numerous advantages associated with BTV subunit vaccination, it has not been commercialized. This is due to the following drawbacks associated with this strategy: a) although high expression levels of the BTV VP2 are possible, it seems that 90% of the expressed protein is insoluble. The soluble VP2 fraction may serve as an appropriate vaccine (results of trials discussed

above), however the quantity expressed is a limiting factor and b) while VLPs may seem like the ideal vaccination strategy, this approach is curbed by the difficulty experienced with large scale expression as well as a reduced stability upon storage (Roy, Urakawa et al. 1990; Pattenden, Middelberg et al. 2005; Lubroth, Rweyemamu et al. 2007).

4.4.1. Possible mode of action of a BTV-VP2 subunit vaccine

A vaccine exploits the memory capacity of the immune system thereby allowing the immune system to rapidly circumvent future infections by the same or similar pathogens which it (the vaccine) represents. Therefore the closer vaccine resembles the authentic pathogen the more rapid and effective will the neutralizing response be toward the pathogen. The vaccine should target the adaptive immune system stimulating both humoral and cellular responses (Shams 2005).

For the purposes of this project, BTV will be considered in terms of how a hypothetical vaccine utilizing outer capsid protein VP2 may function. BTV infection and subsequent disease proceeds via three steps (explained above). Ideally inhibition to any one of these steps might circumvent a viral infection and corresponding disease. Since antibodies raised against BTV-VP5 did not neutralize BTV, we will consider the mode of action of a BTV-VP2 vaccine (Huisman, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Lobato, Coupar et al. 1997; Forzan, Wirblich et al. 2004). The VP2 protein will be taken up by APCs as well as nucleated host cells. The concept of vaccinology is dependent on a humoral response which in turn depends on MHC class II presentation of VP2. The VP2 will be degraded into small peptides known as antigens (short for antibody generator) and presented via MHC class II in APCs. MHC class II will activate CD4⁺ T_h cells via the CD4 surface receptor. This in-turn causes the proliferation and differentiation of the T_h cells into either T_h1 or T_h2 cells. The reasoning behind which T_h cell is produced is unknown. T_h1 cells produces interferon-gamma, activates macrophages, stimulates the B-cells to produce opsonizing antibodies and stimulates cytotoxic CD8⁺ T-cells (CTL-cell-mediated immunity). Not all T_h1 cells are involved in the above. Some form memory T-cells. T_h2 cells are responsible for the release of interleukin 4 and stimulation of B-cells to divide and differentiate into antibody producing plasma cells as well as memory B-cells (humoral immunity). The memory B and T-cells are important since they maintain their affinity for the original antigen and form the basis of a vaccine (Minichiello 2002; Janeway, Travers et al. 2005). When challenged with a BTV, the following may prevent infection and disease:

- i. Antibodies specific for VP2 are in circulation. These antibodies bind the viral surface protein (neutralize the virus) making them unavailable for host receptor binding and simultaneously opsonizing the viral particles which are then taken up by phagocytes.

- ii. Memory B and T-cells interact with APC presenting VP2 in association with MHC class II. The memory B and T-cells are then immediately able to carry out humoral and cellular responses (describe above) respectively circumventing various time-consuming steps such as T_h cell differentiation and proliferation, ergo preventing infection and disease.

Nucleated host cells will present degraded VP2 peptides associated with MHC class I. This activates CTL response. The cytotoxic CD8+ T-cells kill off virally infected cells which is necessary if the virus evades the humoral immunity and infects the host cells (Chattergoon, Robinson et al. 1998).

5. *IN VITRO* EXPRESSION OF ANTIGENIC PROTEINS

In order to produce a proteins vaccine, it is necessary to take various factors into consideration such as the nature/properties of the antigen and the protein expression system that is able to produce large amounts of the antigen for commercial purposes. Discussed below are the various aspects that are taken into consideration prior to vaccine production.

5.1. Protein folding (An overview)

The molecular structures of living systems display a remarkable ability to self assemble with commitment and precision. Protein folding into its intricate three-dimensional functional unit is perhaps at the basis of such a statement. The importance of such a process is manifested in the resulting, highly specific structures which allow for their biological activity. The correctly folded structures also confer stability of the protein in the surrounding environment as well as allow for selective interactions with partner polypeptides. It should be mentioned that a protein in its native conformation with buried hydrophobic regions and exposed hydrophilic residues is referred to as a soluble protein (Dinner, Sali et al. 2000; Dobson 2003). The process of protein folding has only recently become elucidated and was found not to consist of fixed points between specific folded states but trial and error toward the most thermodynamically favorable state (Dobson and Ellis 1998). Therefore a polypeptide chain undergoes a stochastic search of possible conformations, allowing highly separated residues in an amino acid sequence to reach close proximity of each other. In essence the native residual interactions are more stable and thus more persistent thus allowing the lowest-energy structure to be assembled. The trial and error approach toward a stable structure would theoretically be a time consuming task however the energy state is defined by an amino acid sequence. This inevitably means that *in vivo*, natural selection favored proteins that are prone to rapid and efficient folding. Therefore the protein undergoes only a small array of possible conformations before resulting in its native conformation (Wolynes, Onuchic et al. 1995; Dobson 2003). Previous studies involving modeling of protein folding through energy states have been

imperative in elucidating the mechanism through which a protein achieves its lowest energy state. The model funnels the primary structure of a protein molecule (approximately 100 residues) toward a so called “saddle point”, which is a transition the molecule must pass in order to reach the native state. At the transition, interactions are limited to a small number of key residues which forms a native-like folding nucleus around which the rest of the structure condenses forming the native protein topology (Onuchic, Socci et al. 1996; Dinner, Sali et al. 2000; Dobson 2003). So in summary the interaction of key residues forces the polypeptide chain toward a native structure. This in turn suggests that the protein fold is determined primarily by the hydrophobic and polar residues that interacts preferentially and favors only specific residues as the protein moves toward its lowest energy state (Vendruscolo, Paci et al. 2001). With regard to the larger proteins, it seems that folding takes place independently in different regions of the protein in which the key residues establish a native-like fold within and between the protein domains. This is followed by a cooperative folding step where the protein side chains are interact forming a unique closed arrangement from which water is excluded. This model is particularly attractive since it implies that a complex protein is built in manageable pieces (Dinner, Sali et al. 2000; Dobson 2003).

5.2. *In vitro* protein expression systems

Over the years much research has been invested into the design of an expression system which not only allows high level of protein production but also the recovery of such proteins in a soluble form. Various systems i.e. prokaryotic, baculovirus-insect cell, mammalian and yeast systems have been developed to allow for the expression of foreign proteins (Verma, Boleti et al. 1998).

Despite the many advances in cellular machineries for recombinant protein synthesis, prokaryotic expression systems lie at the forefront of any expression strategy. This system provides an inexpensive, rapid approach toward high level protein expression for both research and commercial purposes (Verma, Boleti et al. 1998; Singh and Panda 2005). Gram negative *Escherichia coli* remains one most favorable choices for heterologous protein production as it is very well established. The plasticity of this expression system allows for various manipulations in terms of the choice of cellular compartment for the expressed protein. Whereas the cytoplasmic environment is too reducing, the periplasm provides an oxidizing environment with a variety of enzymes such as disulfide binding protein to assist with disulphide bond formation (Joly and Swartz 1994; Georgiou and Segatori 2005). However the 530 amino acid therapeutic-grade human tissue plasminogen activator consisting of 17 disulfide bonds which is produced commercially was refolded from cytoplasmic inclusion bodies (Georgiou and Valax 1996; Misawa and Kumagai 1999).

A downside to bacterial expression systems are the lack of a post-translational modification pathways such as the glycosylation found in eukaryotes (Georgiou and Valax 1996).

Baculovirus expression in insect cells such as *Spodoptera frugiperda* (Sf) is widely used in the expression of viral proteins. This system also allows for high level of recombinant protein expression however with one marked advantage over bacterial expression systems i.e. the capacity to perform many post-translational modifications such as glycosylation, phosphorylation, acylation and palmitylation (Mikhailov, Monastyrskaya et al. 1996; Roy, Mikhailov et al. 1997; Verma, Boleti et al. 1998).

The application and success of bacterial and baculovirus-Sf9 expression systems in vaccinology are discussed under Section 4.3.4. Subunit vaccines.

Mammalian expression systems although linked to production of soluble protein due to the presence of an efficient post-translational modification system, protein production is slower. Also the requirement of specialized media to maintain mammalian cells makes this system particularly expensive (Verma, Boleti et al. 1998).

The yeast system is advantageous since it is both a microorganisms as well as a eukaryote. It allows for rapid high level protein expression on simple media. Thus it provides an alternative inexpensive method of recombinant protein expression. Unlike bacterial systems, yeast allows for glycosylation of expressed proteins at the Asn-X-Ser/Thr motif. The insert DNA encoding the foreign protein is integrated into the yeast genome thus ensuring stability for many generations. While the yeast system has been associated with production of properly folded proteins, soluble proteins, inclusion bodies do occasionally form as does degradation of improperly folded proteins (Verma, Boleti et al. 1998).

5.3. Problems experienced during *in vitro* protein expression

During *in vitro* synthesis of non-native proteins, single domain proteins undergo rapid and efficient folding, thereby burying exposed hydrophobic residues. This is rarely the case for larger proteins with multiple domains (Dobson and Karplus 1999). Since protein folding is dictated by the amino acid constitute of the polypeptide chain, nascent polypeptide chains can only begin folding once translation is complete and the peptide exits the ribosome as the size constraint of the ribosomal unit hinders protein folding within it. Therefore as the chain is being synthesized and exits the ribosome, non-native interactions may produce misfolded intermediates (Frydman 2001; Hartl and Hayer-Hartl 2002). The misfolded intermediates are however stable enough despite exposed hydrophobic (sticky) regions and tend to self associate as well as interact with other misfolded or partially folded

(discussed below) proteins through hydrophobic and interchain hydrogen bonding. This eventually leads to protein aggregation which irreversibly removes a protein from the productive folding pathway (Radford 2000; Hartl and Hayer-Hartl 2002). A high local concentration of nascent chains drives interactions between misfolded states which further promotes aggregation. This in turn suggests that a common deposition of the over-expressed proteins may in fact promote aggregation (Hartl 1996). Kinetic barriers are another important consideration during *in vitro* synthesis of non-native proteins since covalent reactions such as the formation of disulphide bonds by cysteine residues, cis or trans isomerization around the proline peptide bonds and the pre/post-protein processing are a necessity (Wetzel 1994; Georgiou and Valax 1996). The formation of disulfide bonds is a two step process, firstly requiring the oxidation of cysteine residues followed by isomerization, a slow rate-limiting step during folding of cysteine-rich proteins (Idicula-Thomas and Balaji 2005). High level protein synthesis in the presence of such kinetic barriers would eventually lead to the inefficiency of the host chaperones and folding catalyst thereby resulting in the deposition of partially folded intermediates with exposed hydrophobic surfaces in the cytosol. These partially folded intermediates may be considered as near-native conformations of the recombinant proteins. However, once again these exposed hydrophobic patches promote self association of the folding intermediates leading to insoluble protein aggregations or inclusion bodies in bacterial expression systems (Georgiou, Valax et al. 1994). In addition to the above, protein aggregation may also be the direct result of an unsuitable local environment for disulfide bond formation, the lack of a post-translational machinery and with regard to the amino acid constituent of a protein, a high degree of hydrophobic residues and the limited solubility associated with such proteins (Georgiou and Valax 1996; Doglia, Ami et al. 2008). Thus far, it seems that a protein aggregation is composed of partially folded and misfolded intermediates. However proteins in its native conformation i.e. soluble proteins may also accumulate within the aggregation (Baneyx and Mujacic 2004). Due to the common cellular localization of the different protein states together with the macromolecular crowding within the cell which promotes compaction and association, it seems logical that soluble proteins may also accumulate within the aggregation. The soluble proteins by themselves will display a distinct sedimentation pattern; however within an aggregation, the soluble proteins will sediment together with aggregation as explained by mass action (Horn and Jackson 1972).

5.4. Recovery of soluble proteins by chemical treatment

Protein aggregates are often frowned upon; however such aggregations offer various advantages. The constituent proteins are chiefly the expressed recombinant proteins and thus enable convenient purification of the protein of interest. The storage of the proteins within the aggregates protects against proteolytic cleavage by host proteases as well as offers the option of high level expression of

toxic proteins since aggregated proteins are usually inactive (Singh and Panda 2005; Jungbauer and Kaar 2007).

The aggregation of proteins is a completely reversible process by chemical treatment post harvest of the expressed proteins. The constituent proteins may be disaggregated or solubilized and refolded (Misawa and Kumagai 1999; Singh, Eshwari et al. 2005).

Arginine, a polar amino acid was found to play an important role in the suppression of aggregation. However it does not refold proteins (Arakawa and Tsumoto 2003). In 2003, Tsumoto, et al. showed that arginine also disaggregated green fluorescent protein (GFP) which then fluoresced. The arginine treatment released native GFP from an insoluble aggregation (Tsumoto, Umetsu et al. 2003). The mechanism behind the action of arginine is yet to be elucidate however it is thought that the interaction between the guanidine structure of arginine and tryptophan residues of proteins assist in the suppression of aggregation (Fischer, Sumner et al. 1993; Rudolph and Lilie 1996; Singh and Panda 2005). With regard to its disaggregating activity, this positively charged residue ($pI > pH$) surrounds the proteins in the aggregation causing a repulsive force which in turn disaggregates the proteins (Henk Huismans, personal communication).

Denaturation may be carried out using denaturants such as strong chaotropic reagents (urea or guanidine-hydrochloride) or detergents (SDS or sarkosyl). These reagents lead to the unfolding of the protein such that they assume a flexible and disordered structure in solution. Ionic detergents form strong electrostatic repulsion around the proteins i.e. detergent/protein complexes and therefore seem to be most efficient at the dispersion of an aggregation into monomolecular structures (Takagi and Kubo 1979; Gierasch, Lacy et al. 1982; Tsumoto, Ejima et al. 2003). In 2003, Tsumoto, et al. showed that a low concentration of guanidine-hydrochloride ($< 6\text{ M}$) could solubilize fluorescent green fluorescent protein (Tsumoto, Umetsu et al. 2003). Reducing reagents such as β -mercaptoethanol and dithiotreitol (DTT) prevent the formation of non-native disulphide bonds by maintaining cysteine residues in a reduced state (Tsumoto, Ejima et al. 2003).

The proteins that are in a flexible, unfolded and solvated state may then be refolded (Vallejo and Rinas 2004; Singh and Panda 2005). Refolding refers to the change in the conformation of proteins toward a native, stable state. This process may be carried out the removal of the denaturants and the addition of a suitable refolding buffer which in essence causes the proteins to fold into its native conformation (Tsumoto, Ejima et al. 2003). However the complete removal of the denaturants may force protein to undergo aggregation once more. Therefore the refolding approached should be optimized such that proteins maintain a degree of solubility and flexibility while refolding (Singh, Eshwari et al. 2005). It is for this reason that a refolding buffer usually dilutes out the denaturant as

well as contain additives such as oxidized and reduced glutathione. The oxidized glutathione forms disulphide bonds with the denatured proteins followed by the refolding process aided by catalytic amounts of glutathione in the reduced form (Shi, Wang et al. 2006).

5.5. Protein folding with molecular chaperones

Albeit foreign proteins have the propensity to aggregate during high level production, various expression systems have cellular chaperone machinery which counteracts aggregation. Such machinery prevents misfolding of cytoplasmic native proteins and may be exploited to cater to foreign proteins (Georgiou and Valax 1996; Hartl and Hayer-Hartl 2002). Chaperones recognize and bind characteristic features of nascent polypeptide chains such as exposed hydrophobic regions and an accessible peptide backbone thereby shielding these interactive surfaces. This in-turn removes these regions from interacting non-natively and thus through a series of substrate binding and release cycles, these chaperones assist with proper folding of proteins (Fenton and Horwich 2003; Summers, Douglas et al. 2009). Three chaperone sets namely, DnaK-dnaJ-GrpE, GroEL-GroES (GroE) and trigger factor (TF) which assist in the folding of nascent polypeptide chains in the cytosol are discussed (Figure 5).

TF (48 kDa) binds to the ribosome and interacts with short nascent polypeptide chain (< 57 residues) that are rich in aromatic (hydrophobic) residues as the chain exits the ribosome. The interaction stabilizes the nascent chain in a state facilitating proper folding (Hesterkamp, Hauser et al. 1996; Patzelt, Rudiger et al. 2001).

Longer polypeptide chains (proteins > 60 kDa) will subsequently bind the dnaK-dnaJ-GrpE chaperone system which promotes protein folding through a series of ATP-dependent binding and release (Hartl and Hayer-Hartl 2002). dnaJ (40 kDa) recognizes exposed hydrophobic patches on nascent polypeptide chains and recruits dnaK (70 kDa). The ATP-bound dnaK binds the peptide chain in its peptide binding cleft through hydrophobic side chain interaction and hydrogen bonds on peptide backbone (Zhu, Zhao et al. 1996). The alpha-helical latch over the cleft undergoes a conformational change thus closing the latch. This conformational change occurs by the hydrolysis of ATP to ADP due to the binding of dnaJ to dnaK. This results in the stable holding of the peptide. GrpE (20 kDa) facilitates the release of ADP from the dnaK and allows for dnaK to once again bind ATP, thereby disassociating the dnaK-peptide complex (Harrison, Hayer-Hartl et al. 1997; Hartl and Hayer-Hartl 2002). The idea behind this mechanism is that multiple binding of dnaK to polypeptide chain prevents non-native interactions thus preventing intramolecular misfolding (Bukau and Horwich 1998).

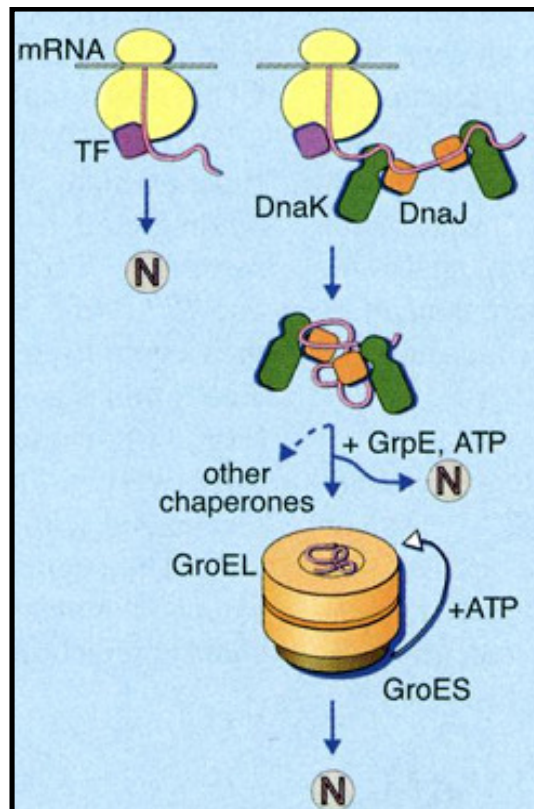


Figure 5. Model of DnaK-dnaJ-GrpE, GroE and TF assisted protein folding in the cytosol. TF is involved with protein folding of smaller nascent polypeptide chains (minimum 57 residues). Longer polypeptide chains interact with the DnaK-dnaJ-GrpE chaperone set through a series of ATP-dependent binding and release cycles which prevents non-native interactions and promotes native protein (N) folding. Proteins that are less than 60 kDa may transit through the GroEL-GroES chaperonin system. This system encloses the polypeptide within a 'barrel' which provides a suitable environment for protein folding (Hartl and Hayer-Hartl 2002).

The GroE chaperone system assists in protein folding of non-native proteins up to 60 kDa (Brinker, Pfeifer et al. 2001). GroEL contains two identical 57 kDa heptameric rings stacked back-to-back. The exposed hydrophobic apical domain of the trans ring of the GroEL interacts with exposed hydrophobic amino acids on polypeptide chains. This is followed by the binding of seven ATP molecules to the equatorial GroEL domain and the binding of the homoheptameric GroES comprising a ring of 10 kDa subunits at the GroEL end thereby enclosing of the polypeptide within the GroEL. This results in the disassociation of the ATP and GroES from the cis ring of the GroEL (Hartl 1996; Roseman, Chen et al. 1996; Xu, Horwich et al. 1997). The apical domain of the trans ring of the GroEL then undergoes a rotation and upward movement which results in the enlargement of the cavity and a hydrophilic shift in the surface (Xu, Horwich et al. 1997). The hydrophilicity causes a release of the polypeptide which is now free within the GroEL-GroES complex and can undergo proper folding for approximately ten seconds before the hydrolysis of the ATP molecules.

Upon the completion of hydrolysis of ATP, the ADP and GroES is released triggering the opening and release of the native or non-native protein. The non-native protein is once again captured by the chaperone system (Hartl and Hayer-Hartl 2002).

6. AIMS OF THE CURRENT STUDY

The safety concerns associated with BTV live-attenuated vaccine (highlighted above) and the global spread of BTV highlight the importance for the development of a new generation vaccination strategy (Veronesi, Hamblin et al. 2005). BTV outer capsid protein VP2 by itself has been implicated in previous studies as the main neutralization-specific antigen and despite the lack of an atomic structure to identify immunogenically exposed epitopes; studies have described the position of the neutralizing epitopes as confined to the N-terminal of the protein (+/- first 450 amino acids) (Huismans, van der Walt et al. 1987; Inumaru and Roy 1987; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; Athmaram, Bali et al. 2007). The use of BTV-VP2 as a subunit vaccine requires that this antigen is expressed in a correctly folded and soluble state. Solubility refers to an ordered tertiary structure whereby the neutralizing epitopes are displayed to the immune system as found on the cognate protein (Dinner, Sali et al. 2000; Dobson 2003). Solubility is important since the immune response toward insoluble or aggregated proteins is generally poor. Aggregation is often the result of non-native interactions which occur within and between newly synthesized polypeptide chains. Previous studies have shown that aggregation can be overcome by generating truncated versions of a particular antigen which contains the major neutralizing epitopes but lack hydrophobic regions which promote aggregation (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

The long-term aim of this project is the development of a subunit vaccine against BTV using the outer capsid protein VP2 or hydrophilic truncations of this protein which overlaps regions containing neutralizing epitopes that are predicted from literature. The aim of this study more specifically was the evaluation and comparison of solubility of full-length BTV(10)-VP2 and truncated versions of the protein synthesized in both a prokaryotic and a baculovirus-Sf9 expression system. To address the aim, this study was focused on the following:

1. Evaluation of the solubility of prokaryotic and baculovirus-Sf9 expressed full-length BTV(10)-VP2 and truncated versions of this protein that overlap regions containing the previously reported neutralizing epitopes.
2. Optimization of the bacterial expression in order to increase the yield of soluble proteins by means of differential induction conditions, variable expression temperatures and protein harvest times.

3. Co-expression of bacterial molecular chaperones and heat-shock proteins (hsps) with the full-length BTV(10)-VP2 and truncated proteins to assist with synthesis of soluble proteins.
4. Solubilization of BTV(10)-VP2 and truncated BTV(10)-VP2 proteins by arginine treatment of the protein aggregations.

CHAPTER 2: PROKARYOTIC EXPRESSION OF FULL-LENGTH BTV-VP2 AND TRUNCATED VERSIONS OF THIS PROTEIN

1. INTRODUCTION

A number of studies have been focused on the role of BTV-VP2 by itself in eliciting a protective immune response in sheep (Chapter 1, section 4.4; (Huismans and Erasmus 1981; Huismans, van der Walt et al. 1987; Inumaru and Roy 1987; Roy, Urakawa et al. 1990). The commercialization of BTV-VP2 by itself as a subunit vaccine has been hindered by high levels of aggregated and insoluble protein synthesized during *in vitro* protein expression due to non-native interaction between newly synthesized polypeptide chains (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Pattenden, Middelberg et al. 2005; Lubroth, Rweyemamu et al. 2007).

Solubility is very important when considering the use of a single subunit protein vaccine. Solubility refers to the capacity of the expressed antigen to fold into an ordered tertiary structure such that the neutralizing epitopes are exposed to the immune system as found on authentic viral particles (Dinner, Sali et al. 2000; Dobson 2003). The problem associated with the insolubility of the full-length BTV-VP2 may be addressed by generating BTV-VP2 peptides from which some of the major hydrophobic residues have been deleted. The effectiveness of using truncated versions of a particular protein as an antigen was shown to be dependent on inclusion of the essential neutralizing epitopes within the truncated peptide (Chapter 1, section 4.3.4). Although the truncated protein may not necessarily occupy the exact three dimensional structure of the full-length parent protein, a number of studies have shown the strategy to be successful (Fukumoto, Xuan et al. 2003; Bonafe, Rinerger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

When analyzing the BTV-VP2 protein in terms of neutralizing epitopes, the identification of such key exposed regions on the surface of the protein is hampered by the lack of an atomic structure. However various studies demonstrating the protective capacity of the outer capsid protein have complemented each other with a general consensus of the position of the neutralizing epitopes lying approximately within the first 450 amino acids of the full-length VP2 protein (White and Eaton 1990; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; DeMaula, Bonneau et al. 2000; Bhattacharya, Noad et al. 2007; Schwartz-Cornil, Mertens et al. 2008).

Although there are numerous commercial protein expression systems available that can be used to express the BTV-VP2 peptides, there is no formula to predict if the proteins are likely to be soluble or not. Furthermore as the expression systems advance in terms of the technology and associated

success, it may be necessary to revisit these expression systems using their respective new optimizations.

The prokaryotic or bacterial expression system remains an attractive choice for heterologous protein synthesis. This is due to the low cost of this system associated with rapid and high productivity (Terpe 2006). The application and success of the bacterial expression system in vaccinology has been discussed in great detail in Chapter 1, section 4.3.4. However when considering application, it is necessary to mention its shortcomings, the most important being that foreign protein often fail to attain their native three dimensional conformation. This is frequently due to a) the reducing cytoplasmic environment which is unsuitable for the formation of disulfide bonds, b) the overburdening of the host chaperones and folding catalyst during high level protein synthesis or c) the lack of sophisticated post-translational pathways such as glycosylation (as discussed in Chapter 1, section 5.3).

However due to the plasticity of the bacterial expression system, it can be manipulated to allow for the synthesis of a variety of soluble recombinant proteins (Georgiou and Valax 1996) (Chapter 1, section, 5.4 and 5.5). Expression of soluble proteins may be achieved by either an attenuation of expression thereby reducing the propensity for self association and aggregation, the co-expression of molecular chaperones to assist in complete folding of recombinant proteins (Chapter 1, section 5.5) or chemical treatment of the expression products to reverse aggregation (Chapter 1, section 5.4).

Factors controlling recombinant protein expression in a prokaryotic expression system include induction conditions, temperature and protein harvest times. With regard to induction conditions, a decrease in the concentration of inducer (e.g. IPTG) may retard the level of transcription of the target genes thereby decreasing the level of the corresponding protein (Terpe 2006). Temperature is directly correlated to the growth kinetics of bacteria. A decrease in temperature slows down bacterial growth and consequently protein synthesis (Sahdev, Khattar et al. 2008). Induction conditions and expression temperature may prevent the over-burdening of host native chaperones and foldases which can facilitate complete folding of the expressed proteins (Sorensen and Mortensen 2005; Sahdev, Khattar et al. 2008). Protein harvesting time is another important consideration during expression since an early harvest time may circumvent unfavorable protein interactions and protein accumulation which may lead to aggregation (Deacon, Roach et al. 2008).

Molecular chaperones are already operative under physiological growth conditions. However during over-expression of recombinant proteins, the folding machinery becomes over-burdened with the newly synthesized polypeptide chains (Tomoyasu, Mogk et al. 2001; Hartl and Hayer-Hartl 2002).

For longer polypeptide chains, multiple rounds of capture and release by the native chaperones to facilitate complete folding is not possible. This leads to the deposition of partially folded intermediates within the cellular cytoplasm. Often after exiting the ribosomes, polypeptide chains fail to associate with the limited molecular chaperones present thereby causing these chains to misfold (Hartl 1996). The partially folded and misfolded proteins associate by non-native interactions leading to protein aggregation (Georgiou and Valax 1996). An appropriate compensation to the over-burdened host chaperones would be to over-express the appropriate chaperones to cater to the recombinant proteins.

Heat-shock proteins (Hsps) are molecular chaperones that recognize and bind non-native proteins thereby preventing irreversible aggregation. The Hsps family of chaperones is expressed at high levels during heat shock and is involved in thermo-tolerance resulting in cellular survival (Buchner 1996; Diamant, Eliahu et al. 2001). Inclusion body formation is readily observed in *E. coli* mutants lacking the hsp response and similarly inclusion body formation is suppressed by simultaneous over-expression of the hsps. The hsps therefore play a pivotal role in minimizing protein aggregation during *in vitro* over-expression of recombinant proteins (Gragerov, Martin et al. 1991). High external salinity is another type of cell stress which was found to play a role in chaperone regulation and protein folding. Under salt stress, cells accumulate small organic compounds or osmolytes to equilibrate the osmotic pressure (Diamant, Eliahu et al. 2001). When present in low physiological concentrations, osmolytes such as glycine betaine, glycerol, proline, and trehalose function as “chemical chaperones” (Welch and Brown 1996). They assist in local folding of chaperone-polypeptide complexes and stabilize native proteins at the end of the chaperone reaction (Diamant, Eliahu et al. 2001).

A comprehensive review of the various chemical treatment strategies that reverse aggregation or assist in protein denaturation and refolding may be found in Chapter 1 under section 5.4.

The above serves as a baseline rationale for the prokaryotic expression of BTV-VP2 and truncated versions thereof. In the following chapter, the solubility of BTV-VP2 and truncated BTV-VP2 proteins expressed in a prokaryotic expression system will be investigated. The chapter will also describe a) optimization of protein expression conditions to determine its effect on the recombinant protein solubility; b) modification of the expression system in terms of molecular chaperones and c) recovery of soluble recombinant protein by chemical treatment. The results of this study are imperative for future consideration of the bacterial system for the synthesis of BTV-VP2 based proteins in BTV vaccine development. South African BTV isolate serotype 10 will be used for the investigative studies since it is one of the viral strains in circulation.

2. MATERIALS AND METHODS

2.1. Design of truncated BTV(10)-VP2 proteins

The hydrophobicity distribution of the amino acid sequence of South African BTV isolate serotype 10 (GenBank: AJ585131.1) was analyzed using Kyte and Doolittle prediction (1982) with a window width value of 51 in ANTHEROT 2000 V6.0 (Kyte and Doolittle 1982). The sliding window width value refers to the collective hydrophobicity score of a particular number of amino acids. The hydrophobicity score was plotted as a function of the amino acid number with the scores representing highly hydrophilic (-60) and highly hydrophobic (60). A window width value of 51 produced a global depiction of the overall hydrophobicity of BTV(10)-VP2. Since the N-terminal (first half of BTV-VP2) was associated with lower hydrophobicity than the C-terminal, two truncated BTV-VP2 proteins were designed based on incorporation of the N-terminal neutralizing epitopes and deletion of the C-terminal hydrophobic region i.e. VP2(aa650) incorporating amino acid 1 to 650 and VP2(aa450) incorporating amino acid 1 to 450.

2.2. Polymerase chain reaction (PCR)

The full-length BTV(10)-VP2 DNA in pGEM-Teasy vector was supplied by Dr A. C. Potgieter at Onderstepoort Veterinary Institute (OVI). The DNA fragments encoding full-length BTV(10)-VP2 and the truncated versions thereof were amplified by PCR using appropriate primers listed in Table 2 in a 2720 Thermal Cycler PCR system (Applied Biosystems). The primers were designed with Primer Design 4, version 4.20 and synthesized by Integrated DNA Technologies (IDT) (USA). The forward and reverse primers contained either start or stop codons in the correct reading frame respectively in order to accommodate protein fusion tags where applicable. Three additional nucleotides were added to each primer to stabilize the primer ends. The restriction enzyme sites included in the primers was merely for insert verification and orientation determination reasons.

The following PCR reaction and conditions were used irrespective of the PCR amplicon size: The PCR reaction was carried out in a total volume of 50 µl and contained 32.5 µl Sabex ultra high quality water (UHQ), 10 µl 5x Phusion HF Buffer (including MgCl₂), 200 µM each dNTP, 0.5 µM of the appropriate primer (Table 2), 12 ng template DNA (pGEM-BTV(10)-VP2) and 1 U Phusion DNA Polymerase. The order in which the reagents were added was vitally important due to the 3'-5' exonuclease activity of the Phusion DNA Polymerase which can degrade the primers in the absence of dNTPS and the reaction mixture was prepared on ice. The reaction mixture was subjected to the following PCR cycling conditions: 30 seconds at 98°C for the initial denaturation which was followed by 25 cycles of denaturation at 98°C for 5 seconds, primer annealing at 55°C for 30 seconds and elongation at 72°C for 45 seconds and a final extension step performed at 72°C for

15 minutes. Two controls were included with all PCR reactions i.e. a negative control (no DNA was added) and positive control (previously isolated pGEM-BTV(10)-VP2 construct).

Phusion DNA Polymerase produces blunt ended products which are unsuitable for TA cloning required by the Invitrogen™ vectors used in this project. Thus in order to facilitate efficient ligation of the PCR product with the 3' deoxythymidine overhang of the pCR®8/GW/TOPO® vector used in this project, the gel purified PCR products (eluted in 30 µl UHQ, method below) was added to a second PCR reaction mixture containing Taq polymerase. The terminal transferase activity of Taq polymerase adds a 3' deoxyadenosine overhang to the PCR amplicon during the final PCR extension step. The 50 µl PCR reaction contained 10.75 µl UHQ, 5 µl 10 x TaKaRa Ex Taq Buffer, 2.5 mM each dNTP, 2.5 U Takara Ex Taq™ and 30 µl PCR product. The extension was carried out in the 2720 Thermal Cycler PCR system (Applied Biosystems) at 72 °C for 90 minutes.

Table 2. Primers used to amplify BTV(10)-VP2(ORF), BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) by PCR for the current study. Restriction sites within the primer sequence are highlighted.

PCR product	Primer storage name	5'-3' sequence orientation
BTV(10)-VP2(ORF)	BTV(10)-VP2-XhoI-Fw	GGC C TCGAGATGGAGGAGTTCGTC (Forward)
	BTV(10)-VP2-BglII-Rv	GGA A GATCTCTAAACATTTAGTAG (Reverse)
BTV(10)-VP2(aa650)	BTV(10)-VP2-XhoI-Fw	GGC C TCGAGATGGAGGAGTTCGTC (Forward)
	BTV(10)-VP2(6)-XbaI-Rv	GGCT C TAGACTAATCATTGCGG (Reverse)
BTV(10)-VP2(aa450)	BTV(10)-VP2-XhoI-Fw	GGC C TCGAGATGGAGGAGTTCGTC (Forward)
	BTV(10)-VP2(4)-XbaI-Rv	GGCT C TAGACTAATCATCCTTCTGTGAT (Reverse)

2.3. Agarose gel electrophoresis

DNA was separated on a 1% (w/v) agarose in 1 x Tris-acetate-EDTA (TAE) (40mM Tris-HCl, 20mM Na-Acetate, 1mM EDTA, pH 8.5) containing ethidium bromide to a final concentration of 1%. The gel mix was cooled at 4°C for polymerization and thereafter submerged in 1 x TAE buffer. DNA samples were mixed with 1 x Gel Loading Dye (Fermentas™), loaded into the wells of the gel and separated at 100-120 V, maximum current for a maximum 20 minutes. The samples were thereafter visualized under UV light and each DNA fragment was identified by molecular weight by comparison to λ /EcoRI/HindIII DNA Ladder (Fermentas™) which was run alongside the samples.

2.4. DNA purification

DNA purification was carried out using High Pure PCR Purification Kit (Roche) as per the manufacturer's standard protocol as follows:

The PCR product was separated on 1% agarose gel electrophoresis (method above) and thereafter excised using a scalpel under minimal UV light exposure in order to prevent UV damage. The gel slice containing the DNA is mixed with a DNA Binding Buffer (0.5 M potassium acetate (pH 4.2), 4 M guanidine hydrochloride) and exposed to a temperature of 65°C for 15 minutes in order to melt the agarose and release the DNA. The solution containing the DNA and melted agarose was then passed through High Pure Filter Tubes by desktop centrifugation (16 000 x g). The filters contain glass fibers fleece which selectively captures and binds DNA. The bound DNA was then washed in 70% ethanol solution to remove contaminating salts and was thereafter eluted in UHQ.

2.5. Preparation of chemically competent cells

Three *E. coli* strains namely DH5 α ™, BL21 Star (DE3) and DH10Bac™ were used. Table 3 summarizes the characteristics of the various strains. The DH5 α ™ and DH10Bac™ strains were obtained from Dr Q Meyer (University of Pretoria) while the BL21 Star (DE3) strain was obtained from Dr V van Staden (University of Pretoria). Chemically competent DH5 α ™ and BL21 Star (DE3) cells were prepared by treatment with calcium chloride (CaCl₂), a method previously describe by Sambrook and Russell in 2001 while chemically competent DH10Bac™ cells were prepared using a DMSO method as described by Chung and Miller in 1988. With regard to DH5 α and BL21 Star (DE3), a single colony was inoculated into 5 ml sterile Luria-Bertani broth (LB-broth) (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5) and grown overnight at 37°C with shaking (225 rpm). Approximately 1 ml of this culture was sub-cultured into 100 ml sterile LB-broth and grown to mid-log phase (OD₆₀₀ 0.4 - 0.6) at 37°C with shaking. Cells were collected by centrifugation at 6 000 x g for 5 minutes in a Beckman Coulter Avanti® J-E centrifuge using the JS-5.3 rotor and re-suspended in half the original volume of ice-cold CaCl₂ (50 mM). The cells were

centrifuged for a second time using the previous conditions and re-suspended in 1/20th the original volume of ice-cold CaCl₂ (50 mM) (Sambrook and Russell 2001).

For the preparation of competent DH10BacTM cells, the cells were grown to mid-log phase in LB-broth containing 10 µg/ml tetracycline and 50 µg/ml kanamycin sulfate. The cells were then collected by centrifugation (conditions above) and resuspended in 1/10th of the original volume ice-cold TSB (1.6% (w/v) Peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 10% (w/v) polyethyleneglycol, 1 M MgCl₂, 1 M MgSO₄, 5% (v/v) DMSO) (Chung and Miller 1988). All competent cells were stored on ice for an hour prior to transformation.

Table 3. Table summarizing the characteristics of the different *E. coli* strains used in this project.

<i>E. coli</i> strain	Resistance	Purpose	Characteristics
DH5α	none	Plasmid maintenance	Mutation in endonuclease I (endA1) preventing non-specific DNA digestion and allows for cleaner DNA preparations. This strain also is recA1 which suppress unwanted recombination in cloned DNA and deficient in DNA repair.
BL21 Star (DE3)	none	Protein expression	Reduced proteolysis of expressed proteins due to a mutation in the membrane protease VII (ompT-). Deficient of lon in the lon ATPase-dependent protease minimizes degradation to recombinant proteins. Lysogen of phage DE3 and carries the gene encoding T7 RNA polymerase under the control of the IPTG inducible <i>lacUV5 lac</i> promoter
DH10Bac TM	kanamycin sulfate, tetracycline	Production of recombinant bacmid DNA	endA1 and recA1 (above). Contains lacZα complementation factor which allows for efficient recombinant blue/white screening

Adapted from New England Biolabs® Inc. 2010 Technical Reference > Restriction Endonucleases > Genetic Markers.

2.6. Transformation of competent cells

Plasmids were transformed into chemically competent cells using a protocol described by Sambrook and Russel in 2001 as follows:

Approximately 100 µl of chemically competent cells were gently mixed with 100 ng DNA in a pre-chilled glass test tube. The cells were placed on ice for approximately 30 minutes and thereafter heat-shocked at 42 °C for 90 seconds. The cells were then cooled on ice for 2 minutes before being supplemented with 900 µl LB-broth and incubated at 37 °C for 1 hour with shaking (225 rpm). Depending on the transformation efficiency (cfu/µg DNA = [(cfu on control plate)/(ng of control DNA

used]] x $(1 \times 10^3 \text{ ng/}\mu\text{g})$ x (dilution plated)), appropriate amounts of the transformation mixture were plated on agar plates (LB broth + 1.5% (w/v) Agar) supplemented with the appropriate antibiotics for different strains of *E. coli* (Table 3 in current chapter) as well as the transformed vectors.

Bacterial co-expression of with chaperone sets from Takara involved an initial co-transformation method. Chemically competent BL21 Star (DE3) were transformed with the chaperone team plasmid (Table 5) and plated on agar plates containing 20 $\mu\text{g/ml}$ Chloramphenicol. A single transformant containing the chaperone plasmid was selected and treated with calcium chloride (Sambrook and Russell 2001) in order to make the cells chemically competent again (Section 2.7, current chapter). The chemically competent cells were then transformed with the recombinant pET160-DEST plasmid using standard transformation method (as described above) and plated on agar plates containing 100 $\mu\text{g/ml}$ Ampicillin (recombinant pET160-DEST) and 20 $\mu\text{g/ml}$ Chloramphenicol (chaperone plasmid). A detailed method is described in the Takara Chaperone Plasmid Set manual.

2.7. Plasmid isolation

Isolation of plasmids was carried out using High Pure Plasmid Isolation Kit from Roche according to the manufacturer's specifications. The protocol describes the Sambrook and Russell's method of plasmid isolation by alkaline lysis (Sambrook and Russell 2001). Single bacterial colonies containing the recombinant plasmid were inoculated into 8 ml LB broth containing appropriate antibiotics. The culture was maintained at 37°C for 16 hours with shaking (225 rpm). Bacterial cells were collected at 6000 x g for 10 minutes using a Beckman Coulter Avanti® J-E centrifuge using the JS-5.3 rotor. The medium was discarded and the cells re-suspended in 250 μl Suspension buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2.5 mg RNase A) and 250 μl Lysis buffer (0.2 M NaOH, 1% SDS). The lysate was incubated for 5 minutes at room temperature followed by the precipitation of the bacterial proteins upon the addition of 350 μl chilled Binding Buffer (0.5 M potassium acetate (pH 4.2), 4 M guanidine hydrochloride). The mixture was incubated for a further 5 minutes on ice followed by centrifugation at 16 000 x g in an Eppendorf 5415D centrifuge for 10 minutes. The pellet was discarded while the supernatant transferred to a High Pure Filter Tube containing glass fiber fleece which has an affinity for DNA and once again centrifuged at 13 000 x g for 1 minute. The flow-through was discarded while the filter with bound DNA was washed with 500 μl Wash Buffer I (20 mM Tris-HCl (pH 6.6), 5 M guanidine hydrochloride, 70% (v/v) ethanol), centrifuged once again at 13 000 x g for 1 minute and thereafter eluted in UHQ. The concentration of the isolated plasmids was measured with the NanoDrop ND1000 spectrophotometer using ND1000 standard operating protocol and UHQ as a standard.

2.8. Cloning in bacterial expression vector pET160-DEST and baculovirus expression vector pDESTTM8

2.8.1. Cloning into entry vector pCR[®]8/GW/TOPO[®]

The PCR amplified full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) DNA segments were cloned to the pCR[®]8/GW/TOPO[®] vector as per pCR[®]8/GW/TOPO[®] TA Cloning[®] Kit (InvitrogenTM) specifications as follows:

The 6 µl TOPO[®] cloning reaction contained 4 µl of PCR product, 1 µl salt solution (1.2 M NaCl and 0.06 M MgCl₂), 10 ng TOPO[®] vector and UHQ. The reaction was incubated at room temperature for 30 minute thereafter transformed into DH5αTM chemically competent cells and plated on agar (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% NaCl (w/v), 1.5% (w/v) Agar, pH 7.5) containing 100 µg/ml Spectomycin.

The recombinant pCR[®]8/GW/TOPO[®] was isolated thereafter restricted by restriction enzyme digestion and sequenced in order to determine the target gene orientation and to verify sequence homology to that of the BTV(10)-VP2 reference sequence (GenBank: AJ585131.1) respectively.

2.8.2. Cloning into bacterial (pET160-DEST) and baculovirus (pDEST8TM) expression vectors (LR reaction)

pET160-DEST and pDESTTM 8 (InvitrogenTM) were chosen for bacterial and baculovirus-Sf9 expression (used in Chapter 3) respectively. Cloning into both expression vectors was carried out as per the Baculovirus Expression System with Gateway[®] Technology manual from InvitrogenTM as follows:

The 10 µl reaction contained 150 ng entry clone, 150 ng expression vector, 2 µl LR ClonaseTM II enzyme and 1 x Tris-EDTA (1 M Tris-HCl (pH 8.0), 0.5 M EDTA). The reaction was incubated at 25°C for 16 hours. Thereafter 1 µl of Proteinase K was added to the reaction which was incubated at 37°C for a further 10 minutes. The entire reaction mixture was then transformed into chemically competent DH5α and plated on agar plates containing the appropriate antibiotics i.e. 100 µg/ml Ampicillin for recombinant pET160-DEST and 50 µg/ml Gentamicin for recombinant pDEST8TM.

2.9. Restriction endonuclease digestion

Isolated recombinant constructs were restricted using a double digestion method in order to verify the presence of the target gene and to determine its orientation. The 10 µl reaction contained 1 µg DNA, 1 µl 10 x SuRE/Cut Buffer H (Roche), 1 unit EcoRV (Roche), 1 unit XhoI (Roche) and UHQ up to 10 µl. The reaction was incubated at 37°C for 1 hour and thereafter analyzed by agarose gel electrophoresis.

2.10. DNA sequencing of recombinant vectors

Sequencing was carried out using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit™ V. 3.0 (Perkin Elmer, Applied Biosciences) according to the standard protocol. The sequencing reaction containing 200 ng DNA, 2 µl BigDye® Terminator Ready Reaction Mix, 3.2 pmol of the appropriate primer, 1 µl BigDye® Sequencing Buffer and UHQ made up to 10 µl was performed in a 2720 Thermal Cycler PCR system (Applied Biosystems) system using the following cycling conditions: 25 cycles of 96°C for 10 seconds (template denaturation), 50°C for 10 seconds (primer annealing) and 60°C for 4 minutes (extension). In addition to the primers listed in Table 2, vector-specific primers listed in Table 4 were also used for sequencing purposes. The sequencing reaction was then made up to 100 µl with 70% ethanol and spun down at 16 000 x g for 30 minutes in an Eppendorf 5415D desktop centrifuge. The supernatant was discarded and the pellet washed in 250 µl 70% ethanol and spun once more using the above conditions. This step was crucial for the remove excess salts and free nucleotides which may interfere with downstream applications. Finally the sequences were analyzed an ABI Prism™ 310 Genetic Analyzer from Perkin Elmer. Sequence analysis and comparison to the reference BTV(10)-VP2 sequence was performed on Vector NTI 9.1.0 (Invitrogen™) and BioEdit Sequence Alignment Editor.

Table 4. Additional commercially available primers used for sequencing purposes (Boehringer Mannheim)

Primer name	Specific for vector	Sequence (5'-3')
M13 forward	pCR®8/GW/TOPO®	GTAAAACGACGGCCAGT
M13 reverse	pCR®8/GW/TOPO®	AACAGCTATGACCATG
T7 forward	pET160-DEST	TAATACGACTCACTATAGGG

2.11. Bacterial expression of BTV(10)-VP2(ORF), BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450)

Bacterial expression vector pET160-DEST from Invitrogen™ (Figure 6) was used for the expression of the recombinant BTV(10)-VP2 proteins in *E. coli* BL21 Star (DE3) (Table 3). The inserted/target gene is under tight control of an IPTG inducible T7/*lac* promoter which allows for high level expression of the gene of interest as a fusion protein coupled at the N-terminal to a polyhistidine tag and a Lumio™ tag. The target protein is separated from the fusion tags by a Tobacco Etch Virus (TEV) protease recognition site. This means that the target protein may be separated from the

fusion tags by means of a TEV cysteine protease. The dual tags allow for the detection of the expressed proteins by means of Lumio™ Green Detection (Invitrogen™) or immunoblot using anti-polyhistidine tag antibodies/probe. The BTV(10) proteins may also be detected with anti-BTV(10) antibodies and due to the presence of the polyhistidine tag, the recombinant fusion protein can be purified from the bacterial native proteins by use of a metal-chelating resin.

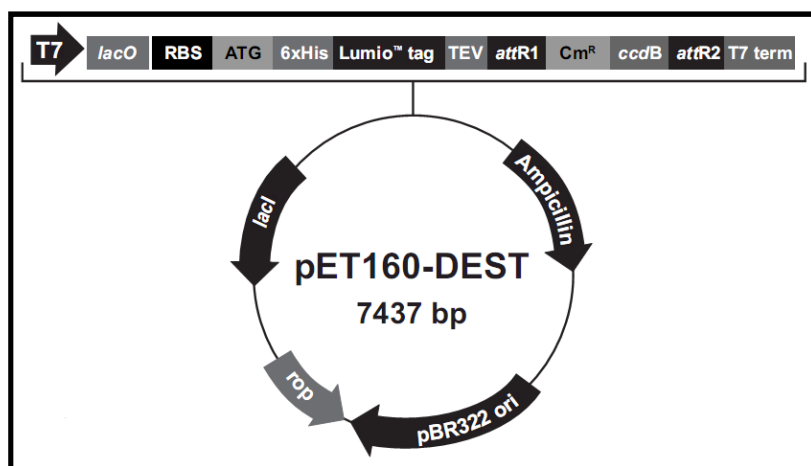


Figure 6. Map of bacterial expression vector pET160-DEST (pET160-DEST manual from Invitrogen™).

2.11.1. Standard bacterial expression protocol

Expression of full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) in protein expression host strain *E. coli* BL21 Star (DE3) was carried as per Champion pET Gateway Expression Kits with Lumio™ Technology manual (Invitrogen™). Any modification to the protocol is amended in the results section of that particular experiment.

The recombinant pET160-DEST plasmid was isolated from *E. coli* DH5α™ and transformed into chemically competent *E. coli* BL21 Star (DE3). A single transformed colony was cultured in 5 ml LB-broth containing appropriate antibiotics for 16 hours at 37°C with shaking (225 rpm). The culture was diluted 1:100 into fresh supplemented LB-broth and grown at 37 °C with shaking (225 rpm) until a specific OD₆₀₀ (as defined by the experiment) was reached. Recombinant protein expression was induced using freshly prepared filter sterilized IPTG in UHQ at a specific concentration dictated by the particular experiment. The induced culture was incubated with shaking (225 rpm) at a predetermined temperature for a predetermined time in accordance to the requirement of the particular experiment. Non-recombinant BL21 Star (DE3) and uninduced recombinant BL21 Star (DE3) which were exposed to the same conditions as the induced culture served as controls.

2.11.2. Co-expression with molecular chaperones

Chaperone co-expression studies were carried out as per the Takara Chaperone Plasmid Set manual as follows:

LB-broth was supplemented with appropriate antibiotics for the chaperone plasmid selection and the recombinant pET160-DEST plasmid selection. Chaperone expression was induced according to the promoter requirements of the chaperone plasmid (Table 5) prior to the induction of expression of the recombinant target protein. The cells expressing the chaperones were allowed to grow and expression of the recombinant proteins was only induced once the cells reached OD₆₀₀ 0.4 – 0.6.

Table 5. Characteristics of the Takara Chaperone Plasmid Set used for this project

Plasmid	Chaperone	Resistance	Promoter	Inducer
pG-KJE8	dnaK-dnaJ-grpE-	Chloramphenicol	araB	L-Arabinose and
	groES-groEL		Pzt1	Tetracyclin
pGro7	groES-groEL	Chloramphenicol	araB	L-Arabinose
pKJE7	dnaK-dnaJ-grpE	Chloramphenicol	araB	L-Arabinose

Adapted from Takara Chaperone Plasmid Set manual

2.11.3. Preparation of bacterial cell lysate

Following expression of recombinant protein, the bacterial cells were collected by centrifugation at 6 000 x g Beckman Coulter Avanti® J-E centrifuge using the JS-5.3 rotor. The culture medium was discarded while the bacterial cells were resuspended in a standard ice cold bacterial cell lysis buffer (50 mM potassium phosphate (pH 7.8), 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole, 1 mg/ml lysozyme (added just before use), 0.7 µg/ml Pepstatin (Roche) and 1mg/ml Pefabloc (Roche)) (100 µl lysis buffer/1 ml culture) and incubated at 30°C for 1 hour. The resulting viscous cell lysate was then sonicated twice using a Branson Sonifier 450 at power output level 3 with constant sonication for 12 seconds and left on ice for 30 seconds between cycles. The lysate was then fractioned and analyzed using methods described under Section 2.12 of the current chapter.

2.12. Protein fractionation and analysis

2.12.1. Protein fractionations methods

2.12.1.1. *High speed centrifugation*

Once the bacterial or baculovirus infected Sf9 cells were lysed, the lysate was centrifuged at 16 000 x g for 30 minutes in an Eppendorf 5415D desktop centrifuge. The supernatant containing soluble proteins was carefully removed and added to a sterile Eppendorf while the pellet fraction containing insoluble proteins was resuspended in an equal volume of 1 x Sodium Chloride–Tris–EDTA buffer (STE) (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) as the supernatant. Approximately 12 µl of each fraction was analyzed by SDS-PAGE and immunoblot.

2.12.1.2. *Sucrose sedimentation gradient*

For experimentation involving the pellet containing the recombinant proteins, the cell lysate was layered on a 2 ml 40% sucrose cushion and centrifuged at 15 000 rpm for 30 minutes with a Beckman SW 22 Ti rotor in Beckman Coulter Ultracentrifuge. A linear discontinuous sucrose gradient was prepared by layering from bottom to top, 800 µl each of 70, 65, 60, 55 and 50% (w/v) sucrose in STE in a 5 ml centrifuge tube. The cell lysate (800 µl) or pellet (untreated or treated with L-arginine to a final concentration of 1.0 M) was then carefully layered on top of the gradient and the gradient centrifuged at 30 000 rpm for 18 hrs with a Beckman SW 22 Ti rotor in Beckman Coulter Ultracentrifuge. Ten fractions of approximately 500 µl were collected using a Minipuls Evolution Fraction Collector (Gilson), each fraction collected from the bottom of the centrifuge tube and labeled 1 to 10. The pellet (P) was resuspended in 500 µl STE buffer. With regard to baculovirus expression in Sf9 cells, 20 µl of each sucrose fraction and pellet was analyzed by SDS-PAGE while due to limited supply of anti-BTV(10) antibodies, only fractions 7-10 were analyzed with immunoblot. For bacterial expression, there was no pellet and 20 µl of each sucrose fraction was analyzed on SDS-PAGE. Due to the extremely high yield of protein synthesis during bacterial expression, identification of proteins could be made on molecular weight negating immunoblot analysis.

2.12.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved under denaturing conditions on a 12% Tris-glycine gel which was thereafter stained with Coomassie blue R-250 in order to visualize the proteins (Laemmli 1970; Sambrook and Russell 2001). The protocol was as follows:

Protein samples were mixed v/v with Reducing Treatment Buffer (125 mM Tris-HCl (pH 6.8), 4% (m/v) SDS, 20% (v/v) glycerol and 10% 9v/v) 2-mercaptoethanol, 0.05% (v/v) Bromophenol Blue in 10 ml UHQ) and denatured at 98°C for 10 minutes. Appropriate amounts were loaded onto a 12%

separating gel (12% polyacrylamide; 0.375 M Tris-HCl, pH 8.8; 0.1% SDS; 0.008% TEMED; 0.08% ammonium persulphate) with 5% stacking gel (5% polyacrylamide; 0.125 M Tris-HCl, pH 6.8; 0.1% SDS; 0.008% TEMED; 0.08% ammonium persulphate) alongside PageRuler™ Prestained Protein Ladder (Fermentas Life Sciences) to estimate the protein molecular weight. Approximately 12 and 20 µl of sample was loaded for crude and sucrose fractionation analysis respectively. SDS-PAGE was conducted at 160 V, 160 A for 3-4 hours on an electrophoresis system from Cleaver Scientific Ltd then stained with Staining Solution (0.125% Coomassie blue R-250, 50% (v/v) methanol, 10 % (v/v) acetic acid in dH₂O) for 30 minutes and destained in 10% (v/v) acetic acid and 50% (v/v) methanol with overnight shaking. For SDS-PAGE analysis of fractions from the sucrose gradient, the Reducing Treatment Buffer was prepared without glycerol which may distort the gel resolution due to the presence of sucrose.

2.12.3. Immunoblot analysis

Subsequent to protein separation by SDS-PAGE (Section 2.14.2), the proteins were transferred by electrophoresis at 100 V and 400 mA for 90 minutes to a Hybond-C+ nitrocellulose membrane (Amersham Biosciences) in Transfer Buffer (0.025 M Tris (pH 8.3), 0.15 M glycine, 20% (v/v) methanol) using a protein immunoblotting system from Cleaver Scientific Ltd. The unoccupied binding sites on the membrane was then blocked with 5% (w/v) non-fat milk in 1 x PBS for 1 hour with shaking. The membrane was then incubated in 15 ml of 1% (w/v) non-fat milk in 1 x PBS containing 72 mg/ml anti-BTV(10) IgY (1 in 250 dilution of 18 mg/ml) (Primary antibody) (Dr W. Fick, University of Pretoria) for 16 hours with shaking at 225 rpm. The antibody was then removed from the membrane which was then washed three times for 5 minutes each in 20 ml Wash Buffer (0.05% (v/v) Tween 20, 1 x PBS) and thereafter incubated with shaking for an hour in a 1/1000 dilution of anti-chicken peroxidase conjugate (Sigma) (Secondary antibody) in 1% (w/v) non-fat milk in 1 x PBS. The secondary antibody was then removed and the membrane washed in 20 ml Wash Buffer (3 times for 5 minutes) and once in 1 x PBS for 5 minutes with shaking. The membrane was then incubated with a Substrate Solution (0.06% (w/v) 4-chloro-naphthol in 20 ml ice-cold methanol mixed with 0.0015% (v/v) hydrogen peroxide in 100 ml 1 x PBS) until the precipitating bands were clearly visible. Thereafter the membrane was washed in distilled water (dH₂O) and dried. The membrane was then scanned using the VersaDoc Imaging System (BioRad).

2.13. Purification of polyhistidine tagged proteins

The purification of the polyhistidine-tagged recombinant proteins was carried out using Protino® Ni-TED kit under native conditions as per the user's manual specifications. The protocol was as follows:

The 5 ml bacterial lysate was added to a 1 x Lysis-Equilibration-Wash buffer (LEW) (50 mM NaH₂PO₄, pH 8.0 and 300 mM NaCl) equilibrated Protino® Ni column which was allowed to drain by gravity. The flow-through was discarded and the column washed twice with 1 x LEW buffer. The recombinant proteins were then eluted three times with 240 µl elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 250 mM imidazole). Samples were collected at each step and approximately 40 µl analyzed with reducing SDS-PAGE to determine where the recombinant proteins eluted.

3. RESULTS

3.1. Design of truncated BTV(10)-VP2 proteins

The amino acid sequence of BTV-VP2 serotype 10 (GenBank: AJ585131.1) was analyzed *in silico* in order to investigate the hydrophobicity distribution relative to the position of the neutralizing epitopes of BTV-VP2. The aim was to design truncated versions of BTV-VP2 that maintained the neutralizing epitopes of the protein (as discussed in the Introduction of the current chapter) but excluded at least some of the hydrophobic regions which could contribute to the insolubility of the expressed proteins.

ANTHEPROT 2000 V6.0 was used to generate the hydrophobicity profile of the South African BTV isolate. The software used a method described by Kyte and Doolittle in 1982 and the prediction was based on a window width value of 52. This gave a global overview of the hydrophobicity distribution of BTV-VP2 (Kyte and Doolittle 1982). The position of the neutralizing epitopes of BTV-VP2 confined to the N-terminal, first 450 amino acids, were then superimposed onto the hydrophobicity profile (Figure 7).

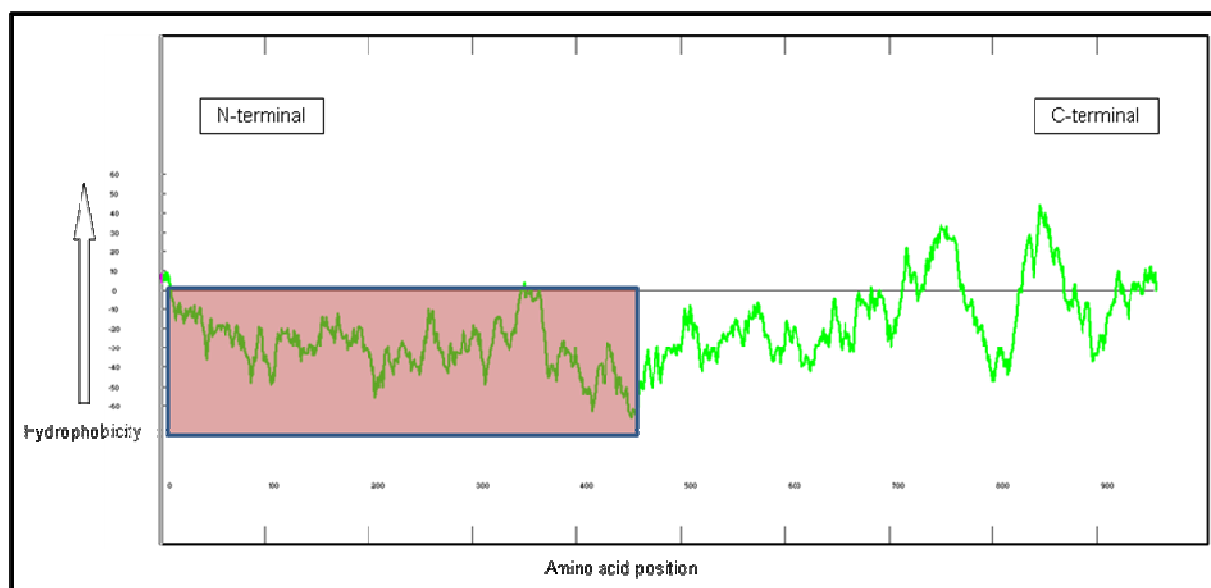


Figure 7. Hydrophobicity distribution of BTV(10)-VP2 generated with ANTHEPROT 2000 V6.0 using Kyte and Doolittle prediction (1982). The amino acid position is indicated on the X-axis while negative values on the Y-axis is indicative of nett hydrophilicity (below the central horizontal line) and positive values on the Y-axis are indicative of nett hydrophobicity (above the central horizontal line). The plot was generated using a window width size of 51. The region containing the neutralizing epitopes of BTV-VP2 is highlighted while the N- and C-terminal of the polypeptide is marked.

The BTV-VP2 N-terminal neutralizing epitopes (+/- first 450 amino acids) overlapped with regions of BTV-VP2 displaying overall lower hydrophobicity compared to the remainder of protein (highlighted

region in Figure 7). Therefore in order to reduce the insolubility of the expressed protein, truncated BTV-VP2 proteins were designed by removal of the C-terminal hydrophobic regions of the full-length BTV(10)-VP2. The two truncated proteins were BTV(10)-VP2(aa650) composed of amino acid 1 to 650 and BTV(10)-VP2(aa450) composed of amino acid 1 to 450 (Figure 8).

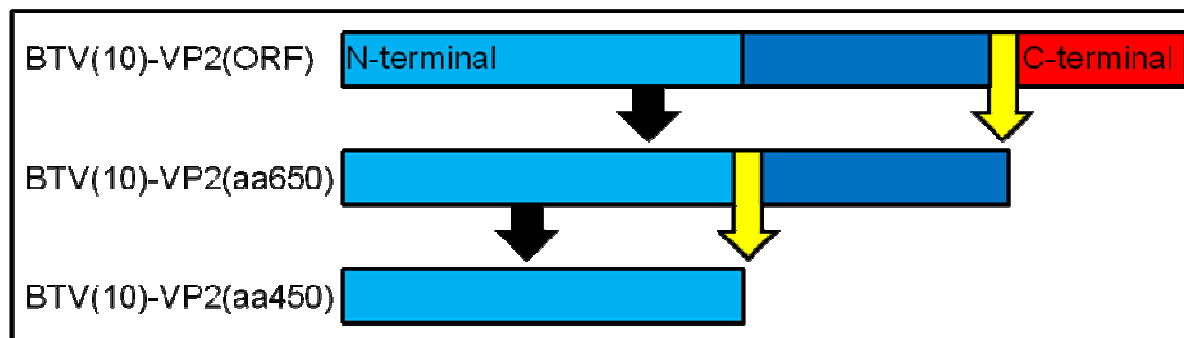


Figure 8. Schematic representation of the truncated BTV(10)-VP2 protein design. C-terminal hydrophobic regions of the full-length BTV(10)-VP2 were removed to produce truncated BTV(10)-VP2(aa450) and BTV(10)-VP2(aa650) proteins. The N- and C-terminal are marked while the yellow arrows denote points where the cut.

The BTV(10)-VP2(aa450) protein was composed predominantly of BTV-VP2 neutralizing epitopes (position of these epitopes discussed in the Introduction of current chapter) while the BTV(10)-VP2(aa650) protein contained in addition to the neutralizing epitopes, a greater portion of the BTV-VP2 backbone. The reason for the design of the BTV(10)-VP2(aa650) protein was that the tertiary conformation of the truncated protein relative to the full-length BTV-VP2 was unknown and a larger truncated protein may provide better options for correct tertiary folding. Although the BTV(10)-VP2(aa650) protein was more hydrophobic than the BTV(10)-VP2(aa450) protein, the hydrophobicity was still lower than the remainder of the full-length BTV-VP2.

3.2. Amplification of full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) by PCR

The genes encoding BTV(10)-VP2(ORF), BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) were amplified by PCR from a template BTV(10)-VP2(ORF) gene in pGEM-Teasy using the gene-specific primers listed in Table 2. The BTV-VP2 template DNA was supplied by Dr A. C. Potgieter at Onderstepoort Veterinary Institute (OVI). A PCR reaction without any template DNA served as a negative control while the positive control reaction contained previously isolated pGEM-Teasy-BTV(10)-VP2 template DNA. The agarose gel-purified PCR amplicons were visualized on a 1% agarose gel stained with ethidium bromide and the genes identified by molecular weight (Figure 9). The theoretical molecular weight of the full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-

VP2(aa450) including the restriction endonuclease sites and additional 'GGC' sequence were 2883, 1974 and 1365 base pairs (bp) respectively.

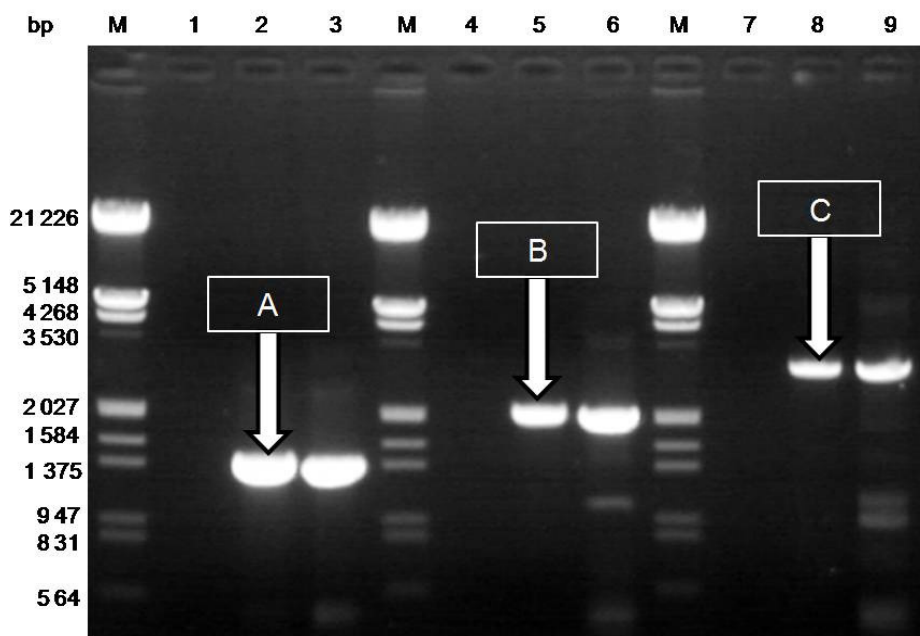


Figure 9. PCR products BTV(10)-VP2(ORF), BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) visualized on a 1% agarose gel stained with ethidium bromide. M, λ EcoRI/HindIII DNA Ladder (Fermentas™); Lane 1, PCR negative control (no template DNA); Lane 2, 1365 bp BTV(10) agarose gel-purified VP2(aa450) amplicon (A); Lane 3, PCR positive control (previously isolated BTV(10)-VP2 template DNA); Lane 4, PCR negative control; Lane 5, 1974 bp BTV(10) agarose gel-purified VP2(aa650) amplicon (B); Lane 6, PCR positive control; Lane 7, PCR negative control; Lane 8, 2883 bp BTV(10) agarose gel-purified VP2(ORF) amplicon (C); Lane 9, PCR positive control.

The agarose gel-purified PCR products as well as the negative and positive PCR control reactions were analyzed alongside molecular weight marker (MWM), λ EcoRI/HindIII DNA Ladder (Fermentas™) (Figure 9). The molecular weight of the PCR amplicons A, B and C (Lane 2, 5 and 8) corresponded to the theoretical molecular weights of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) respectively. The PCR products were subsequently clone into Gateway® pCR®8/GW/TOPO (Invitrogen™) in order to generate a recombinant entry vector.

3.3. Construction of recombinant expression vectors

In order to express the full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) it was necessary to clone the genes into suitable bacterial and baculovirus expression vectors. See Chapter 3 for recombinant baculovirus-Sf9 expression. The cloning strategy for the recombinant bacterial expression vector, pET160-DEST and recombinant baculovirus-Sf9 expression vector pDEST™ 8 (Invitrogen™) is summarized in Figure 10.

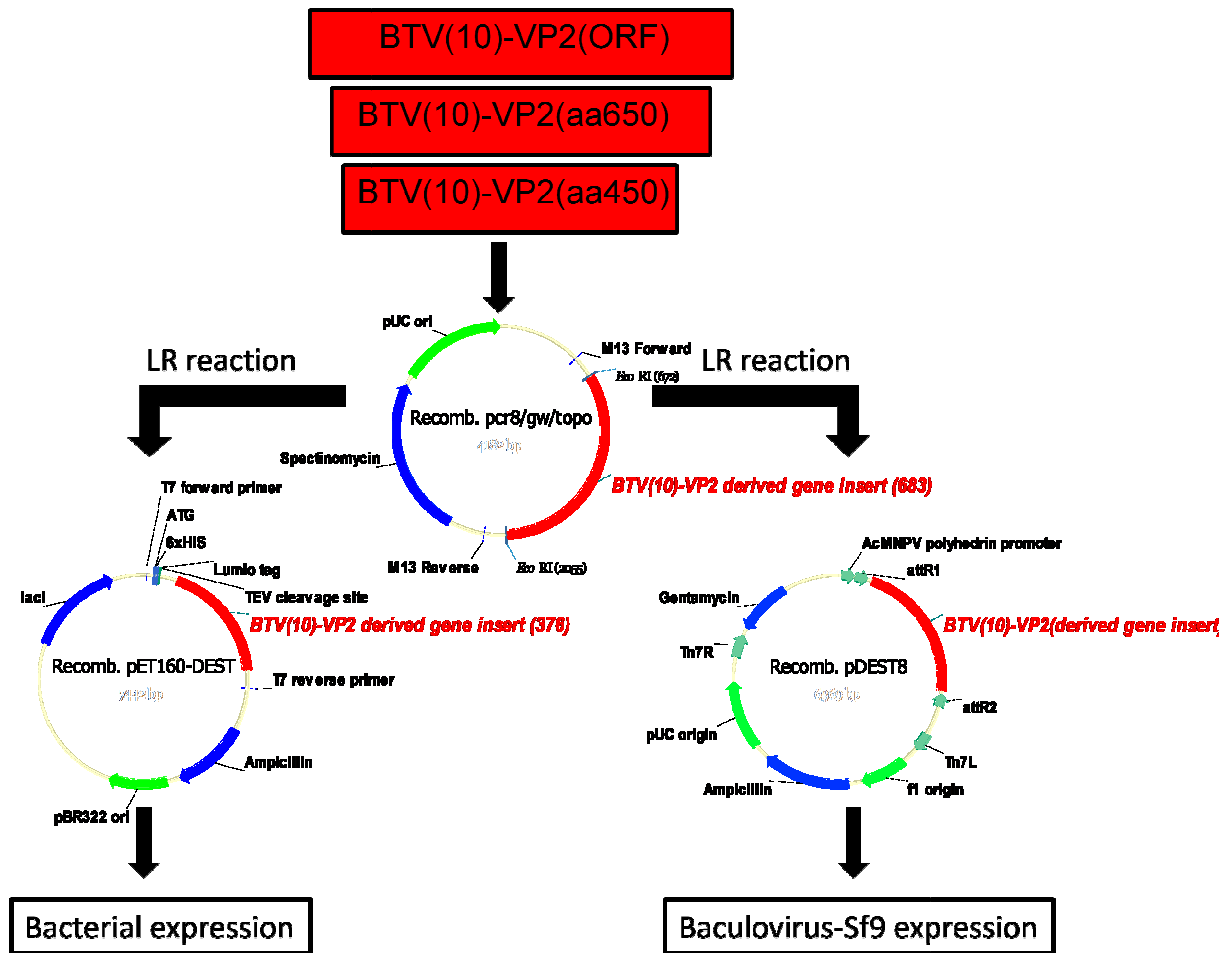


Figure 10. Summary of cloning strategy for full-length BTV(10)-VP2, BTV (10)-VP2(aa650) and BTV(10)-VP2(aa450) into bacterial expression vector, pET160-DEST and baculovirus/Sf9 expression vector, pDEST™ 8 (Invitrogen™). The genes encoding BTV(10)-VP2 and truncations, amplified by PCR, were TA cloned into the Gateway® entry vector pCR®8/GW/TOPO. The genes in the correct orientation verified by restriction digest and sequencing, were then recombined into the respective expression vectors by enzyme LR Clonase™ (Invitrogen™).

3.3.1. Generating an entry clone

The cloning of the insert DNA into expression vectors required an initial cloning of the insert DNA into pCR8/GW/TOPO® vector (Invitrogen™) in order to generate an entry clone. This type of cloning allows for the transfer of the target gene to an appropriate Gateway™ adapted bacterial and baculovirus expression vector from Invitrogen™. The DNA is transferred by a recombination reaction between the entry clone and the expression vector. The genes encoding full-length BTV(10)-VP2 and truncated proteins, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450), were amplified by PCR and cloned into pCR®8/GW/TOPO entry vector (Invitrogen™). The cloning reaction consisted of a ligation between the deoxyadenine overhangs on the PCR product and the

deoxythymidine overhangs on the vector by enzyme Topoisomerase I. The three entry clone reactions were transformed into chemically competent DH5 α TM and grown on agar plates containing appropriate antibiotics. Ten transformed colonies were selected from each of the three transformations and the putative recombinant plasmids were isolated. The isolated plasmids were digested using restriction endonuclease enzymes XhoI (Roche) and EcoRV (Roche). The restricted isolates were then analyzed on 1% agarose stained with ethidium bromide to verify the presence of the insert and determine the gene orientation (Figure 11). Non-recombinant pCR $\oplus$ 8/GW/TOPO restricted with XhoI and EcoRV was used as a positive control while unrestricted pCR $\oplus$ 8/GW/TOPO was used as a negative control. The above restriction digest of the recombinant pCR $\oplus$ 8/GW/TOPO plasmids were replicated *in silico* using Vector NTI 9.1.0 (InvitrogenTM). Theoretically the above restriction yields four fragments for pCR $\oplus$ 8/GW/TOPO-BTV(10)-VP2(aa450) (1446, 1423, 1269 and 44 bp), four fragments for pCR $\oplus$ 8/GW/TOPO-BTV(10)-VP2(aa650) (2055, 1423, 1269 and 44 bp) and four fragments for pCR $\oplus$ 8/GW/TOPO-BTV(10)-VP2(ORF) (2964, 1423, 1269 and 44 bp) when the insert is present in the correct orientation. Non-recombinant pCR $\oplus$ 8/GW/TOPO restricted with XhoI and EcoRV *in silico* yields two fragments of sizes 1548 and 1269 bp.

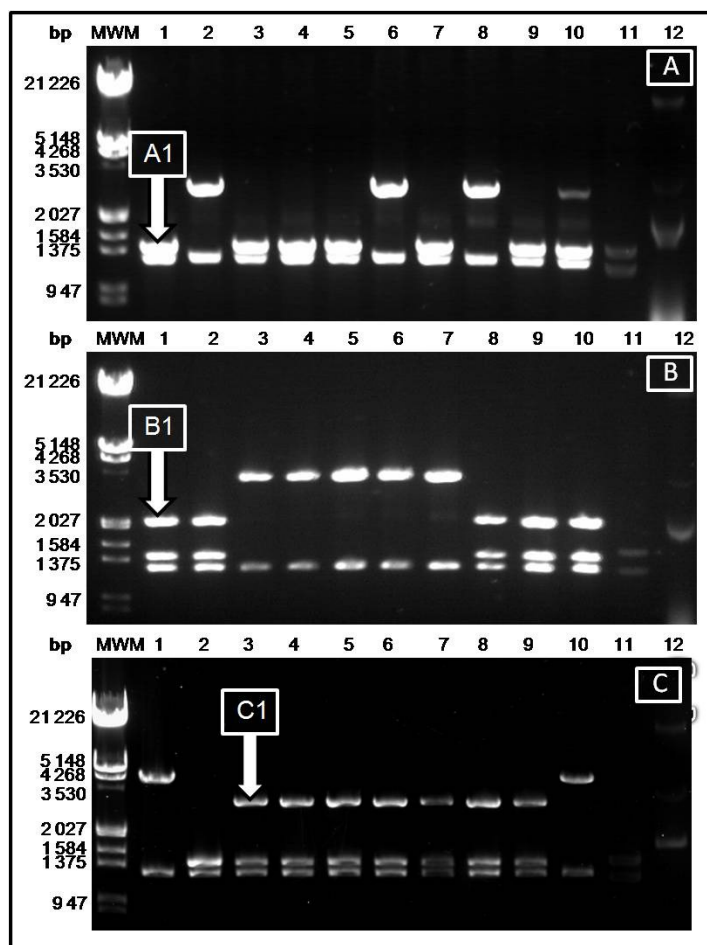


Figure 11. Screening of putative pCR@8/GW/TOPO-BTV(10)-VP2(aa450), pCR@8/GW/TOPO-BTV(10)-VP2(aa650), pCR@8/GW/TOPO-BTV(10)-VP2(ORF) (Panel A, B and C respectively) with restriction endonuclease to verify presence of target gene and to determine gene orientation. MWM, λ EcoRI/HindIII DNA Ladder (Fermentas™); Lane 1-10, putative recombinant pCR@8/GW/TOPO 1-10 restricted with enzymes XhoI and EcoRV; Lane 11, pCR@8/GW/TOPO restricted with enzymes XhoI and EcoRV (positive control); Lane 12, unrestricted pCR@8/GW/TOPO (negative control).

Restriction enzyme digest of putative recombinants (Figure 11), revealed that A1, B1 and C1 (Lane 1 of gel A and B and Lane 3 of gel C respectively) contained restricted DNA fragments that corresponded to the theoretical molecular weights of the *in silico* restricted recombinant pCR@8/GW/TOPO-BTV(10)-VP2(aa450), pCR@8/GW/TOPO-BTV(10)-VP2(aa650) and pCR@8/GW/TOPO-BTV(10)-VP2(ORF) respectively using the same restriction enzymes. Therefore the recombinant plasmids used for A1, B1 and C1 restriction digest contained the target genes in the correct orientation.

The recombinant vectors, pCR@8/GW/TOPO-BTV(10)-VP2(aa450), pCR@8/GW/TOPO-BTV(10)-VP2(aa650) and pCR@8/GW/TOPO-BTV(10)-VP2(ORF), were thereafter sequenced using insert-

specific and vector specific primers (Table 2 and Table 4) to verify the sequence homology to BTV-VP2 serotype 10 (GenBank: AJ585131.1). Sequence analysis conducted with BioEdit Sequence Alignment Editor revealed complete sequence homology to the theoretical BTV(10)-VP2 sequence with correct insert orientation and reading frame (Appendix A).

3.3.2. Cloning into bacterial and baculovirus expression vectors

In order to evaluate the solubility of full-length BTV(10)-VP2 and truncated versions thereof, recombinant expression vectors containing the target gene for the appropriate expression systems were to be constructed. Three recombinant bacterial pET160-DEST expression plasmids and three recombinant baculovirus pDESTTM8 expression plasmids were constructed (Table 6). Although the recombinant baculovirus expression vector was preparation for Chapter 3, the method of construction was the same as the recombinant bacterial expression vector and therefore is included here. The attL sites of recombinant pCR®8/GW/TOPO entry vector were recombined with the attR sites of bacterial expression vector and baculovirus-insect expression vector. This recombination reaction, catalyzed by enzyme Gateway® ClonaseTM II (InvitrogenTM) simultaneously transfers the target gene flanked by the attL sites into the expression vector maintaining DNA insert orientation and gene reading frame. The recombinant expression vectors were transformed into chemically competent DH5 α TM and grown on agar plates containing appropriate antibiotics. Due to the insertional inactivation of the lethal ccdB gene flanked by the attR sites present on both expression vectors, all bacterial colonies possessed a recombinant vector. The ccdB gene encodes DNA gyrase and serves as a positive selection marker by preventing the growth of cells containing non-recombinant vector. However as a precautionary step, the presence of the gene, the gene orientation and reading frame was verified by forward DNA sequencing in the case of the recombinant bacterial expression vectors (Appendix B) and by PCR analysis for the recombinant baculovirus expression vectors (Chapter 2, Section 3.1). Vector specific and gene specific primers were used respectively (Table 3 and Table 2). DNA sequencing of the recombinant bacterial expression vectors was necessary to confirm the presence of the DNA insert as well as the correct reading frame of the polyhistidine tag and LumioTM tag with the target gene. The sequence analysis was conducted in BioEdit Sequence Alignment Editor (Appendix B). Once the sequence of the target BTV(10)-VP2 genes within the pET160-DEST expression plasmid was verified, the genes could be expressed.

A total of nine recombinant plasmids were generated for this project. All recombinant plasmids were labeled according to its commercial backbone vector (Table 6).

Table 6. Recombinant expression plasmids generated and corresponding names.

Backbone vector	DNA Insert	Recombinant vector designated name
pET160-DEST	BTV(10)-VP2(aa450)	pET160-DEST-BTV(10)-VP2(aa450)
(bacterial expression)	BTV(10)-VP2(aa650)	pET160-DEST-BTV(10)-VP2(aa650)
	BTV(10)-VP2(ORF)	pET160-DEST-BTV(10)-VP2(ORF)
pDEST™8	BTV(10)-VP2(aa450)	pDEST™ 8-BTV(10)-VP2(aa450)
(baculovirus expression)	BTV(10)-VP2(aa650)	pDEST™ 8-BTV(10)-VP2(aa650)
	BTV(10)-VP2(ORF)	pDEST™ 8-BTV(10)-VP2(ORF)

3.4. Bacterial expression of recombinant BTV(10)-VP2 proteins

Bacterial expression was used for rapid and high level production of the recombinant BTV(10)-VP2 proteins. The pET160-DEST expression vector from Invitrogen™ was chosen for the bacterial expression of full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450). Expression of the target gene cloned into the vector is under the control of an IPTG inducible T7 λ c promoter which allows for the cytoplasmic expression of the target protein coupled to an N-terminal polyhistidine tag and a Lumio™ tag. The theoretical molecular weight of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) is 51, 66 and 110 kDa respectively. However due to the N-terminal fusion tags of the pET160-DEST, the expected molecular weight of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) was 55, 70 and 114 kDa respectively.

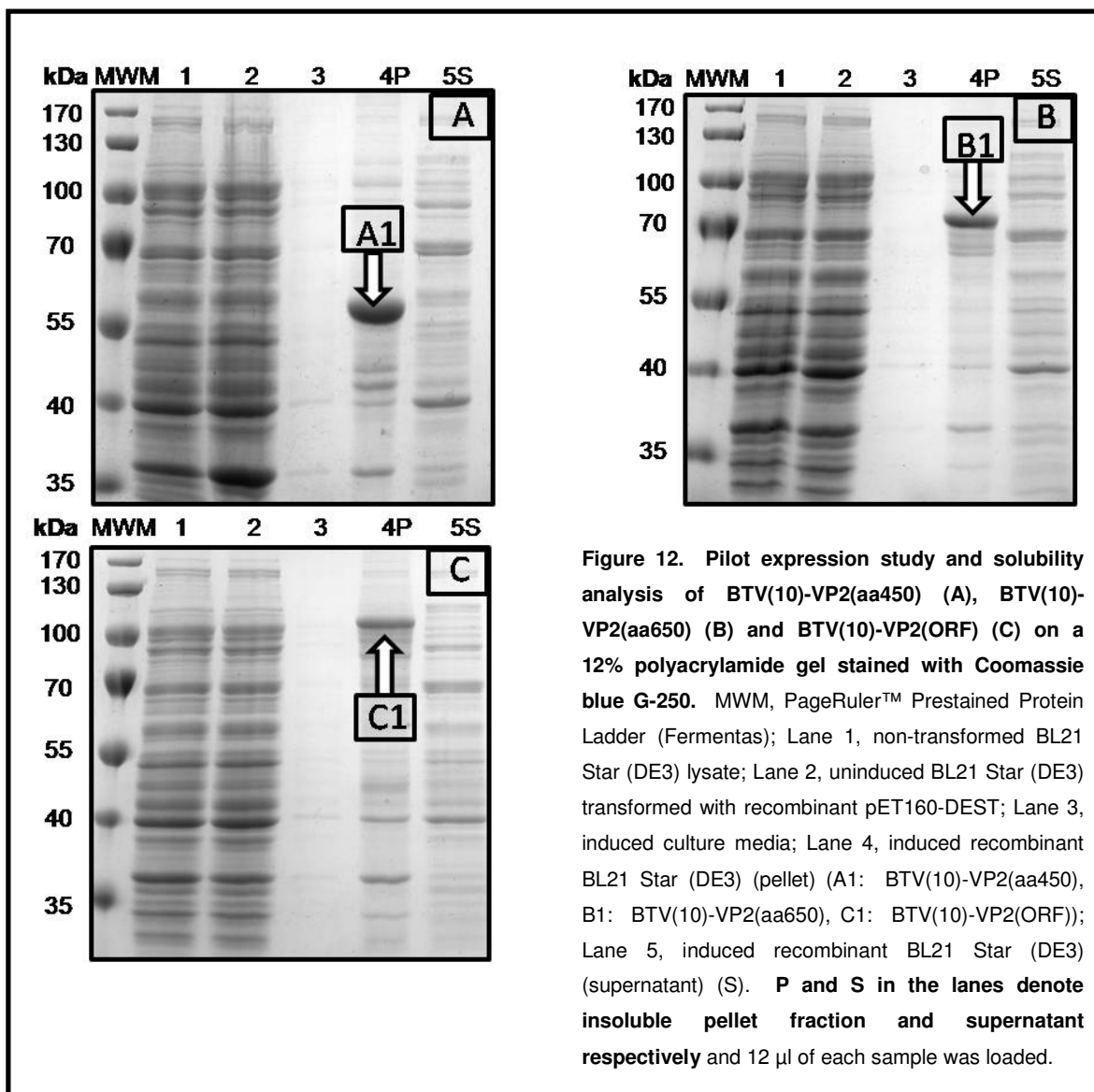
The isolated recombinant pET160-DEST plasmids (Section 3.3.2, current chapter) were transformed into chemically competent *E. coli* expression strain BL21 Star (DE3) (Table 3) and plated on agar plates containing appropriate antibiotics. The next step was to perform an initial pilot expression to confirm expression of the recombinant proteins.

3.4.1. Pilot expression of recombinant BTV(10)-VP2 proteins

The pilot expression study was conducted to confirm recombinant protein expression and evaluate the solubility of BTV(10)-VP2(ORF) and truncated versions thereof.

Protein expression was carried out in the *E. coli* BL21 Star (DE3) expression strain. The solubility of the recombinant proteins was assessed by high speed centrifugation which separates the soluble proteins from the insoluble proteins. A single recombinant colony was selected and grown at 37°C

in LB-broth containing appropriate antibiotics until OD₆₀₀ 0.4 - 0.6. Expression was then induced with 1.0 mM IPTG and continued for 4 hours. Non-transformed BL21 Star (DE3) and uninduced recombinant BL21 Star (DE3) were used as controls. The cells were harvested 4 hours after induction, lysed and the insoluble and soluble proteins separated by high speed centrifugation. The insoluble and soluble fractions were analyzed on 12 % Laemmli SDS polyacrylamide gel stained with Coomassie G-250 (Figure 12).



Proteins A1, B1 and C1 in Lane 4P of Panel A, B and C corresponded to the theoretical molecular weight of the BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) proteins respectively (Figure 12). These recombinant proteins were not seen in non-transformed BL21 Star

(DE3) lysate (Lane 1) and were only found in the insoluble fraction in Lane 4 (P = Pellet). The T7/*lac* promoter exhibited tight regulation over expression since the target proteins were not expressed prior to induction with IPTG (Lane 2). The BTV-VP2 based proteins were not present in the soluble fraction (S = Supernatant) (Lane 5) nor was it exported out of the cell into the culture media (Lane 3).

Immunoblot analysis was carried out to confirm the identity of expressed proteins in the pilot study (Figure 13). Moreover due to the high sensitivity associated with immunoblot (Dennis-Sykes, Miller et al. 1985), it was used to determine whether the soluble fraction contained nanogram (ng) quantities of the target proteins. After PAGE, the proteins were blotted onto a nitrocellulose membrane. Anti-BTV(10) IgY antibodies was used as a primary detection antibody and BL21 Star (DE3) was used as a negative control.

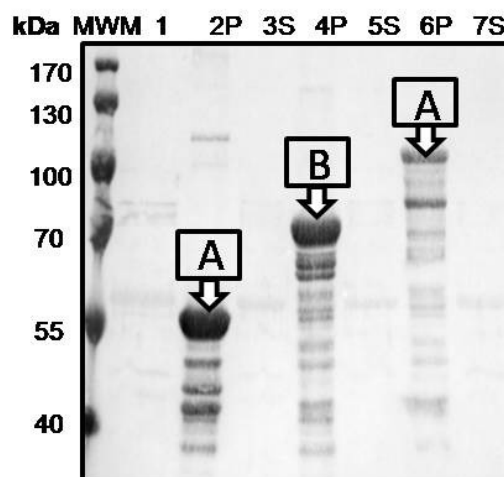


Figure 13. Confirmation of identity of expressed BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) by immunoblot using anti-BTV(10) IgY antibodies. MWM, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, BL21 Star (DE3) lysate; Lane 2, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa450) (pellet); Lane 3, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa450) (supernatant); Lane 4, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa650) (pellet); Lane 5, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa650) (supernatant); Lane 6, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(ORF) (pellet); Lane 7, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(ORF) (supernatant). **P and S in the lanes denote pellet fraction and supernatant respectively** and 12 µl of each sample was loaded.

Immunoblot analysis using anti-BTV(10) IgY antibodies detected three proteins A, B and C in Figure 13 which corresponded once again to the theoretical molecular weight of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) respectively thus confirming their identity.

The analysis also revealed that all expressed proteins were found in the insoluble fraction after high speed centrifugation. The immunoblot was based on linear epitope detection and not conformational since the proteins were firstly resolved on a denaturing polyacrylamide gel. The antibody did not

exhibit cross-reactivity with BL21 Star (DE3) native proteins within the same size range as the recombinant proteins (Lane 1). Furthermore the antibody did not detect any recombinant proteins in the induced soluble fraction (Figure 13, Lane 3, 5 and 7) indicating there were no soluble recombinant BTV(10)-VP2 proteins being expressed. The antibody did however repeatedly show cross-reactivity against proteins of a smaller molecular weight than each expressed protein. Such proteins were probably proteolytic degradation products of the recombinant proteins since it was not present in the supernatant and BL21 Star (DE3) lysate.

Since the recombinant proteins were insoluble, it was necessary to optimize the conditions directly impacting on protein synthesis to investigate the resulting effect on the solubility of these proteins.

3.4.2. Optimization of protein expression conditions (inducer vs. temperature vs. protein harvest)

During high level expression of foreign proteins, the host native chaperones and folding enzymes are overburdened. This leads to partially folded and/or misfolded proteins with exposed hydrophobic regions. These proteins tend to interact with each other resulting in aggregation (Rinas, Hoffmann et al. 2007). If the level of protein expressed was decreased, the host folding pathway would not be overburdened and newly synthesized polypeptide chains could potentially be completed folded. Therefore the optimization of certain variables that impact on the level of protein expression could result in the synthesis of soluble recombinant proteins. Three different factors that affect the level of protein expression namely induction conditions, expression temperature and harvesting times were investigated to determine the effect they might have on the solubility of the expressed recombinant proteins.

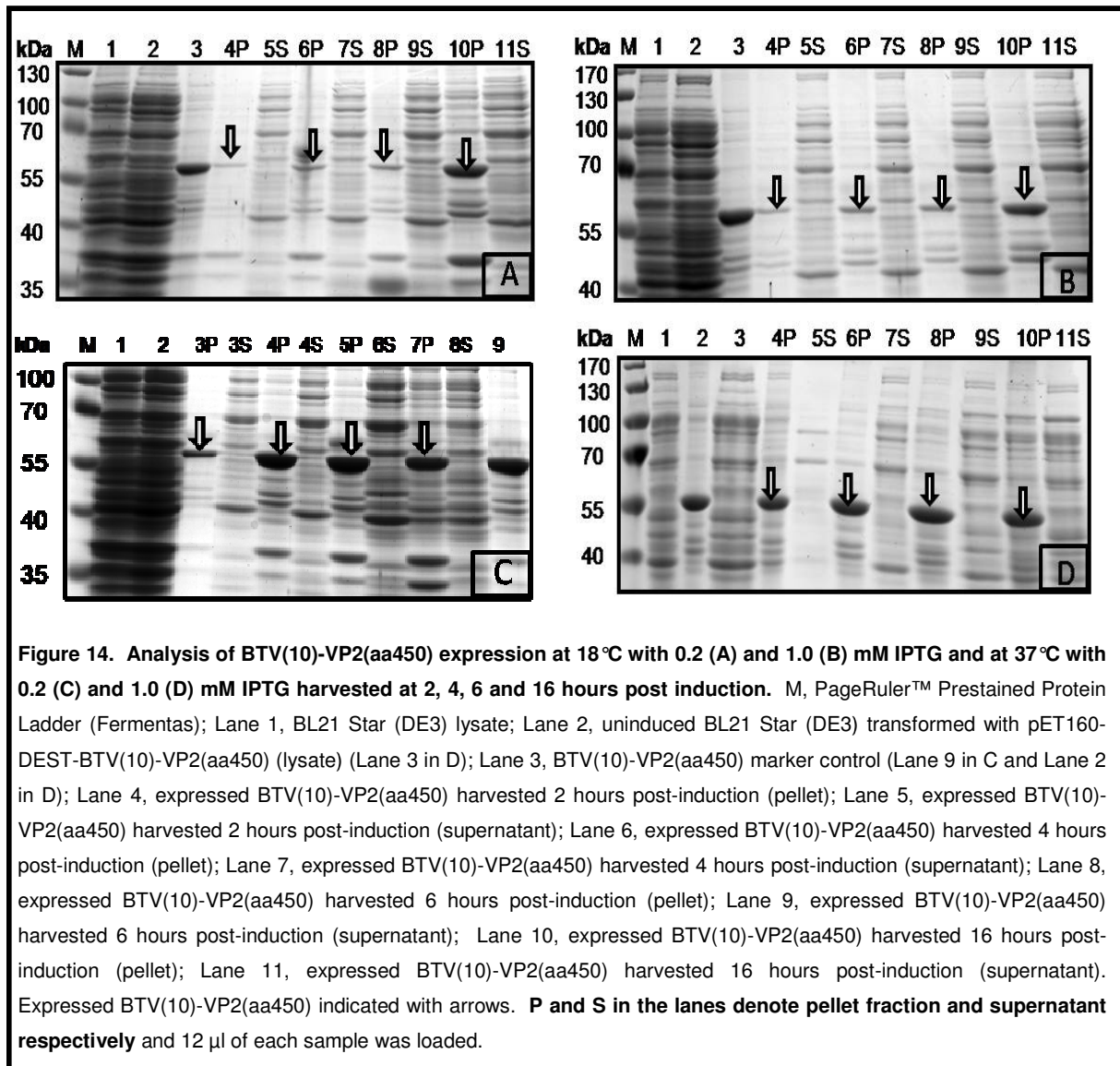
Table 7 summarizes the different expression conditions under which protein expression was conducted in order to investigate the resulting effect on protein solubility. The expression conditions were based on previous studies reporting expression of soluble recombinant proteins (de Marco and De Marco 2004; Luan, Qiu et al. 2004; Deacon, Roach et al. 2008; Sahdev, Khattar et al. 2008). These conditions included expression temperature, 18 °C vs. 37 °C, inducer concentrations, 0.2 mM vs. 1.0 mM IPTG and harvesting times, 2, 4, 6 and 16 hours.

Table 7. Differential expression conditions investigated to determine their effect on protein solubility.

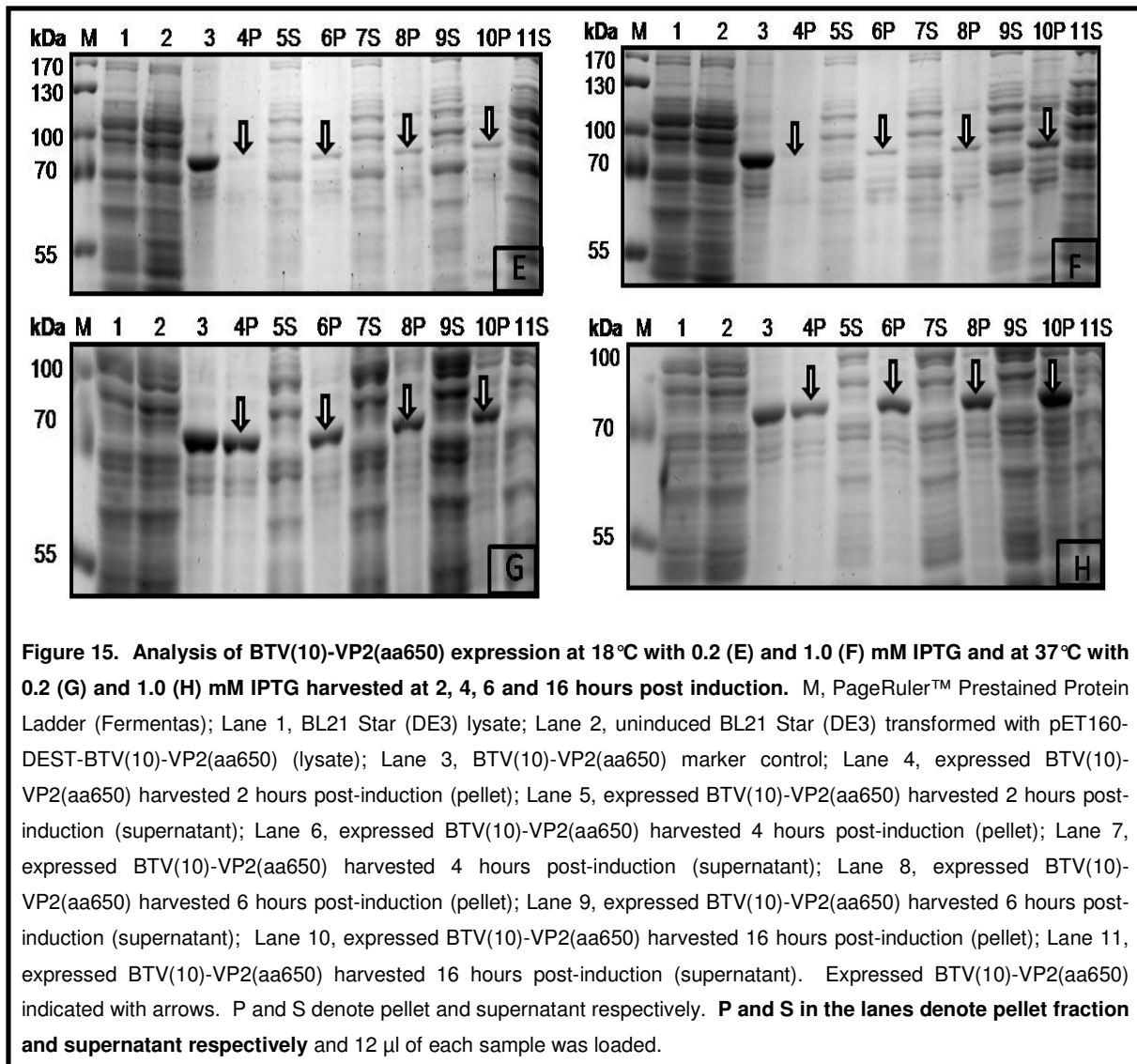
Construct	Expression temperature	Inducer concentration	Harvest time
BTV(10)-VP2(aa450)	18 °C	0.2 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa450)	18 °C	1.0 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa450)	37 °C	0.2 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa450)	37 °C	1.0 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa650)	18 °C	0.2 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa650)	18 °C	1.0 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa650)	37 °C	0.2 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(aa650)	37 °C	1.0 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(ORF)	18 °C	0.2 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(ORF)	18 °C	1.0 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(ORF)	37 °C	0.2 mM IPTG	2, 4, 6, 16hrs
BTV(10)-VP2(ORF)	37 °C	1.0 mM IPTG	2, 4, 6, 16hrs

Following the above expression conditions, the cells were harvested, lysed and the proteins separated by high speed centrifugation in the soluble and insoluble fractions. Non-recombinant BL21 Star (DE3) and appropriate uninduced recombinant BL21 Star (DE3) lysate were used as controls. The soluble and insoluble fractions were analyzed on 12% reducing SDS-PAGE stained with Coomassie G-250. The results of each trial for each recombinant protein are displayed under the respective headings below.

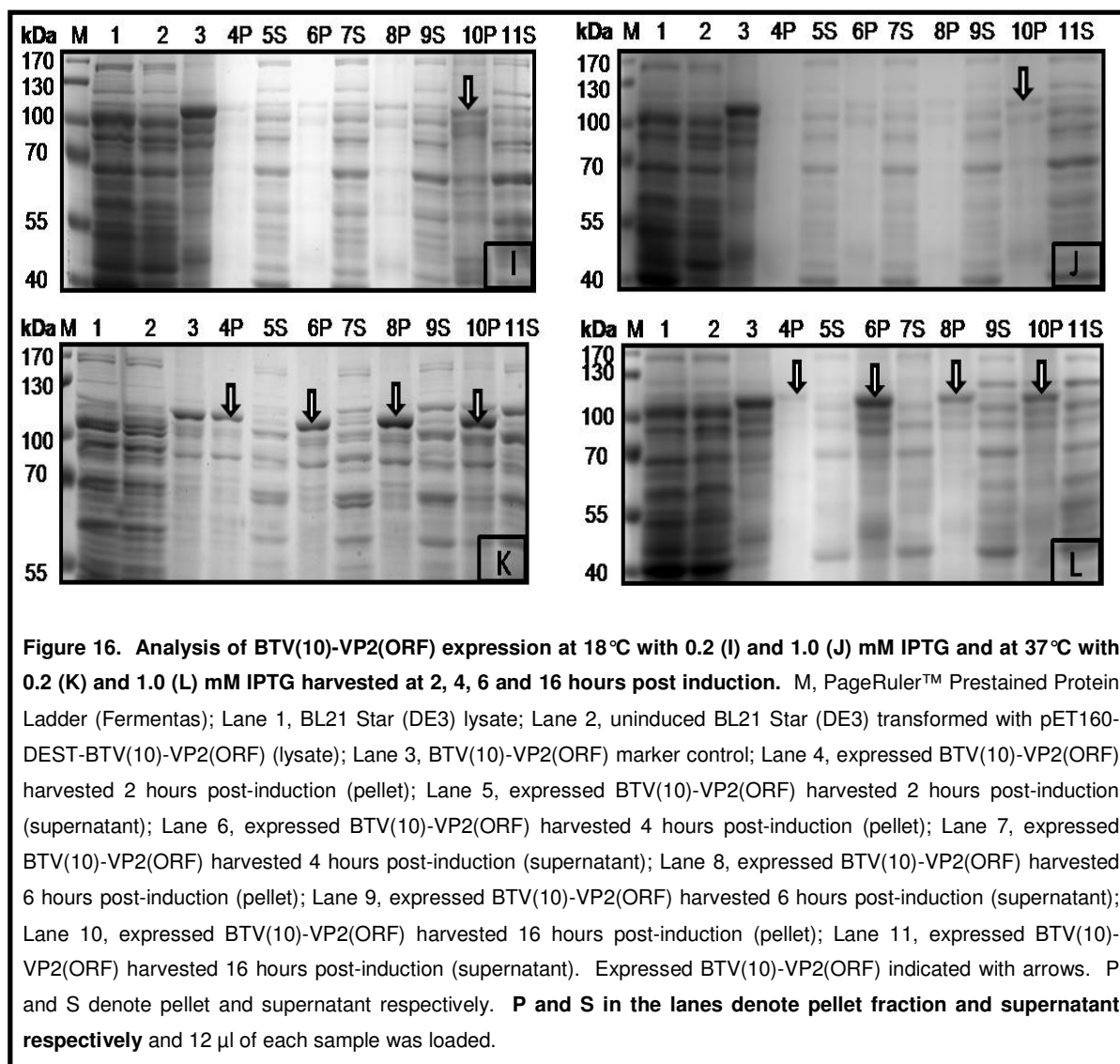
1. BTV(10)-VP2(aa450)



2. BTV(10)-VP2(aa650)



3. BTV(10)-VP2(ORF)



The soluble and insoluble protein fractions were analyzed by means of SDS-PAGE (Figure 14, Figure 15, Figure 16). The results indicated that the expressed BTV(10)-VP2 recombinant proteins remained in the insoluble fraction, indicated by arrows in Figure 14, Figure 15 and Figure 16. Therefore changes in expression inducer (IPTG) concentration, expression temperature and protein harvesting time did not produce soluble recombinant proteins.

The results showed either an increase or decrease in the yield of the recombinant proteins and in the case of full-length BTV(10)-VP2, an absence of protein synthesis under low expression temperature and early protein harvest (Figure 16, Panel I and J, Lane 4, 6 and 8). In general for all of the BTV(10)-VP2 recombinant proteins, a low concentration of inducer, lower expression

temperature and early protein harvest was associated with corresponding low levels of protein synthesis while a high concentration of inducer, higher expression temperature and late protein harvest was associated with corresponding high expression levels of the recombinant proteins.

Since optimization of the expression conditions above did not produce soluble proteins, the recombinant BTV(10)-VP2 proteins were then expressed in host cells over-expressing native heat-shock proteins and chemical chaperones which may assist with the folding of the recombinant proteins.

3.4.3. Expression of the recombinant BTV(10)-VP2 proteins in salt and heat-stressed host cells

The heat-shock proteins (hsps) which are expressed during heat shock bind characteristic features of polypeptide chains such as exposed hydrophobic regions thereby preventing non-native interactions thus circumventing aggregation (Buchner 1996; Diamant, Eliahu et al. 2001). High external salinity plays a role in chaperone regulation and protein folding. Salt-stressed cells accumulate osmolytes which function as “chemical chaperones” to assist in the folding of the chaperone-polypeptide complexes and also stabilize the native proteins at the end of the chaperone reaction (Welch and Brown 1996). (See Chapter 2, Introduction). In the following study, a salt and heat stress environment were combined to induce a native “chemical” and molecular chaperone response in the recombinant BL21 Star (DE3) cells prior to induction of protein expression. In this way the host native chemical chaperones and hsps would be available to assist in folding of the recombinant proteins during high level protein expression.

Single recombinant BL21 Star (DE3) was grown at 37°C to an OD₆₀₀ 0.2 – 0.3. To mimic a salt stress environment, glycerol and L-Glutamic acid mono-potassium salt was added to the cell culture media at a final concentration of 0.1% and 0.1 mM respectively. The cells were heat shocked at 45°C for 30 minutes and then further grown at 37°C until an OD₆₀₀ 0.7 – 0.8. Expression was induced with 1.0 mM IPTG for 4 hours at 37°C. Uninduced recombinant BL21 Star (DE3) cells were used as a negative control to monitor recombinant protein expression. The cells were lysed and the proteins separated into soluble and insoluble fractions by high speed centrifugation. Approximately 12 µl of the insoluble and soluble fraction were then analyzed with SDS-PAGE (Figure 17).

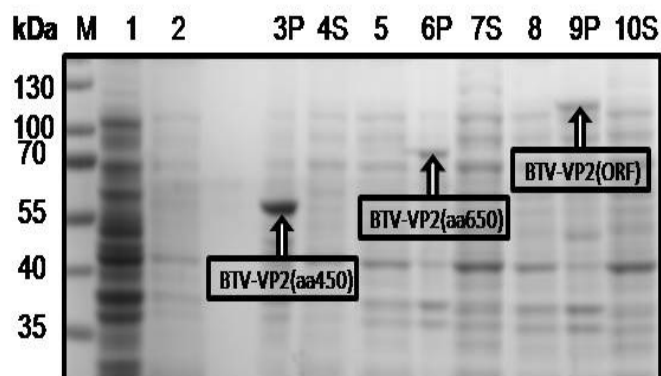


Figure 17. Expression of the recombinant BTV(10)-VP2 proteins in salt and heat stressed BL21 Star (DE3). M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, BL21 Star (DE3) lysate; Lane 2, uninduced BL21 Star (DE3) transformed with pET160-DEST-BTV(10)-VP2(aa450); Lane 3, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa450) (pellet); Lane 4, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa450) (supernatant); Lane 5, uninduced BL21 Star (DE3) transformed with pET160-DEST-BTV(10)-VP2(aa650); Lane 6, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa650) (pellet); Lane 7, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa650) (supernatant); Lane 8, uninduced BL21 Star (DE3) transformed with pET160-DEST-BTV(10)-VP2(ORF); Lane 9, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(ORF) (pellet); Lane 10, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(ORF) (supernatant). **P and S in the lanes denote pellet fraction and supernatant respectively** and 12 µl of each sample was loaded.

The salt and heat-stressed recombinant BL21 Star (DE3) cells did not produce soluble recombinant BTV(10)-VP2 proteins since the proteins were found in the insoluble fraction (Figure 17, Lane 3, 6 and 9 respectively). The yield of recombinant proteins in the salt and heat-stressed cells did however decrease by approximately half in comparison to the original pilot expression study (Section 3.4.1).

Since the over-expression of the host native hsp's and the chemical chaperones did not produce soluble proteins, the next step was to over-express molecular chaperones to assist with folding of the expressed recombinant proteins.

3.4.4. Co-expression of the recombinant BTV(10)-VP2 proteins with molecular chaperones

Molecular chaperones form part of the cellular system responsible for the *de novo* folding of native proteins (Tomoyasu, Mogk et al. 2001). These ubiquitous proteins prevent aggregation, disassemble aggregation and also assist in protein refolding (Diamant, Eliahu et al. 2001). The following set of experiments was based on the exploitation of chaperone-mediated folding pathway to assist in complete folding of the recombinant BTV(10)-VP2 proteins. The recombinant proteins were co-expressed with the Takara Chaperone Plasmid Set (Table 5). The aim here was to over-express the molecular chaperones prior to the expression of the BTV(10)-VP2 proteins which would

then be available to assist in complete/proper folding of these recombinant proteins to native conformation.

3.4.4.1. Co-expression of recombinant BTV(10)-VP2 proteins with chaperones *dnaK*, *dnaJ* and *GrpE*

The *dnaK*-*dnaK*-*GrpE* chaperone system is responsible for the post-translational folding of larger multidomain proteins (> 60 kDa) through a series of binding and release cycles. Multiple *dnaK* binds exposed hydrophobic residues/regions of the polypeptide chain thereby preventing intramolecular misfolding. Upon release, the peptide is free to fold into its native conformation (Bukau and Horwich 1998). In the current study, *dnaK*-*dnaJ*-*GrpE* chaperone system was co-expressed with the recombinant BTV(10)-VP2 proteins to alleviate the over-burdened host native folding machinery and assist with complete folding of the recombinant proteins during high level expression.

Single recombinant colonies containing Takara chaperone plasmid and recombinant pET160-DEST were cultured in LB-broth containing 4 mg/ml L-arabinose. Growth was monitored at 30°C until an OD₆₀₀ 0.4 – 0.6. Protein expression was induced with 1 mM IPTG and continued for 4 hours at 30°C. BL21 Star (DE3) cells expressing only the chaperone were used to assess the chaperone expression. Non-transformed BL21 Star (DE3) cells and uninduced recombinant BL21 Star (DE3) expressing the chaperone set were used as negative controls. After 4 hours, the cells were collected, lysed and the proteins separated once again by high speed centrifugation into soluble and insoluble fractions. The fractions were analyzed with 12% SDS-PAGE stained with Coomassie blue G-250 (Figure 18).

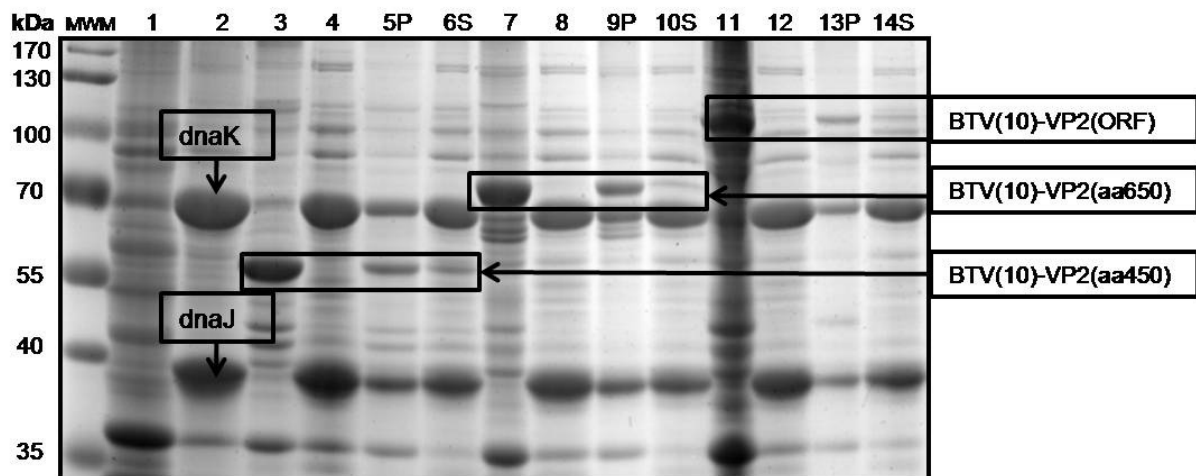


Figure 18. Co-expression of BTV(10)-VP2 recombinant proteins with dnaK-dnaJ-GrpE chaperone system using inducer L-arabinose and IPTG at 4 mg/ml and 1 mM respectively. M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, BL21 Star (DE3) lysate; Lane 2, BL21 Star (DE3) lysate expressing dnaK-dnaJ-grpE only; Lane 3, BTV(10)-VP2(aa450) marker control; Lane 4, uninduced pET160-DEST-BTV(10)-VP2(aa450) in BL21 Star (DE3) expressing chaperone (lysate); Lane 5, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa450) and chaperone (pellet); Lane 6, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa450) and chaperone (supernatant); ; Lane 7, uninduced pET160-DEST-BTV(10)-VP2(aa650) in BL21 Star (DE3) expressing chaperone (lysate); Lane 8, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa650) and chaperone (pellet); Lane 9, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(aa650) chaperone (supernatant); ; Lane 10, uninduced pET160-DEST-BTV(10)-VP2(ORF) in BL21 Star (DE3) expressing chaperone (lysate); Lane 11, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(ORF) and chaperone (pellet); Lane 12, induced recombinant BL21 Star (DE3) expressing BTV(10)-VP2(ORF) and chaperone (supernatant). Chaperone proteins dnaK and dnaJ and regions where the target proteins are found are marked on the figure. **P and S in the lanes denote pellet fraction and supernatant respectively** and 12 µl of each sample was loaded.

The chaperone dnaK and dnaJ were identified by their respective molecular weight and are indicated on Figure 18. The molecular weight of GrpE is 20 kDa and is therefore too small to be detected on the polyacrylamide gel above. However since GrpE is encoded by the same vector as dnaK and dnaJ, its expression was assumed. The recombinant BTV-VP2 proteins were not expressed prior to induction with IPTG and were not present in the uninduced recombinant cell lysates in Lanes 4, 8 and 12. The recombinant proteins were identified by molecular weight and comparison to the recombinant control proteins in Lanes 3, 7 and 11. The recombinant BTV(10)-VP2 proteins were found in both the insoluble fractions (Lanes 5P, 9P and 13P) and the soluble fractions (Lanes 6S, 10S and 14S) after high speed centrifugation. This implied that the dnaK-dnaJ-GrpE chaperones successfully folded a fraction of the recombinant proteins to their respective native conformations, thus aiding in the production of soluble recombinant BTV(10)-VP2 proteins.

With regard to BTV(10)-VP2(aa450) proteins, approximately 50% of the expressed recombinant proteins were present in the soluble fraction (Lane 6S) while approximately 20% of the expressed BTV(10)-VP2(aa650) and full-length BTV(10)-VP2 proteins were found in the soluble fraction (Lane 10S and 14S respectively). These values represent a rough estimate of the recombinant proteins present in the soluble fraction in Figure 18. There was also a decrease in the total yield of the recombinant proteins synthesized as compared to the initial pilot expression study in Figure 12.

The presence of a soluble fraction of the recombinant BTV(10)-VP2 proteins, suggested that these proteins could be purified by virtue of their N-terminal polyhistidine tag. If successfully purified, the vaccine potential of these recombinant proteins could be investigated.

3.4.4.1.1. Purification of the polyhistidine-tagged BTV-VP2 based proteins

The full-length BTV(10)-VP2 and truncated proteins were expressed as fusion proteins tagged with an N-terminal polyhistidine tag. The expression of a soluble fraction of these proteins when co-expressed with the dnaK-dnaJ-GrpE chaperones allowed for purification of native recombinant proteins by immobilized nickel ion affinity chromatography using the polyhistidine tag. The aim here was to purify and concentrate the soluble BTV(10)-VP2 recombinant proteins found in the induced bacterial supernatant.

The bacterial lysate (5 ml) expressing the recombinant protein with chaperone dnaK-dnaJ-GrpE was loaded onto a pre-equilibrated immobilized nickel column (Proino®), washed two times and eluted with imidazole-containing buffer three times. Samples were collected at various stages of the protocol and analyzed with 12% reducing PAGE with was thereafter stained with Coomassie G-250 (Figure 19). Lysate containing the appropriate BTV-VP2 based recombinant protein was used as a marker control.

The fractions eluted during the purification of the recombinant BTV(10)-VP2 proteins were analyzed in Figure 19. Initially the lysates containing the recombinant proteins were separated into insoluble (Lane 2) and soluble (Lane 3) by high speed centrifugation. The supernatant was passed through the nickel column and the flow-through analyzed in Lane 4. The column was then washed twice (Lane 5 and 6) and thereafter eluted three times (Lane 7 – 9). The BTV(10)-VP2 recombinant proteins were present in the supernatant and the wash 1 flow-through (Lane 4 and 5 respectively) while a small quantity of the BTV(10)-VP2(aa450) protein was observed in the wash 2 and elution 1 in Lane 6 and 7 respectively. With a successful purification, the recombinant proteins should only pass through the affinity columns during the final elution steps. However the fact that the BTV(10)-VP2 recombinant proteins passed through the column in the initial flow-through steps is indicative of soluble proteins since particulate proteins cannot move through the column. The inability of the

recombinant proteins to bind the nickel column is suggestive of buried histidine tag residues within the BTV(10)-VP2 recombinant proteins' tertiary structure.

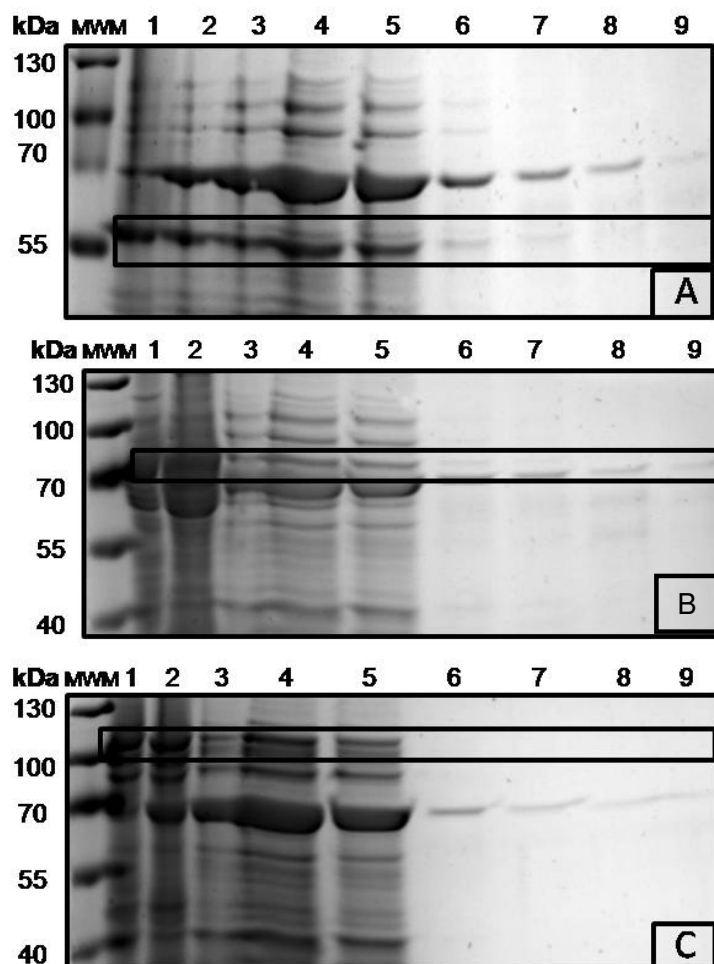


Figure 19. Purification of polyhistidine-tagged BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) using Protino® immobilized nickel affinity chromatography. M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, lysate containing recombinant marker control proteins; Lane 2, induced recombinant BL21 Star (DE3) expressing recombinant protein and chaperone (pellet); Lane 3, induced recombinant BL21 Star (DE3) expressing recombinant protein and chaperone (supernatant); Lane 4, lysate flow-through from column; Lane 5, wash 1 flow-through; Lane 6, wash 2 flow-through; Lane 7, elution 1; Lane 8, elution 2 and Lane 9, elution 3. Boxes indicate position of where the recombinant proteins should lie.

Since the dnaK-dnaJ-GrpE chaperones assisted in the synthesis of a soluble fraction of recombinant BTV-VP2 proteins, the next step was to use these chaperones in combination with the groEL-groES chaperones to determine if this would increase the amount of soluble recombinant proteins.

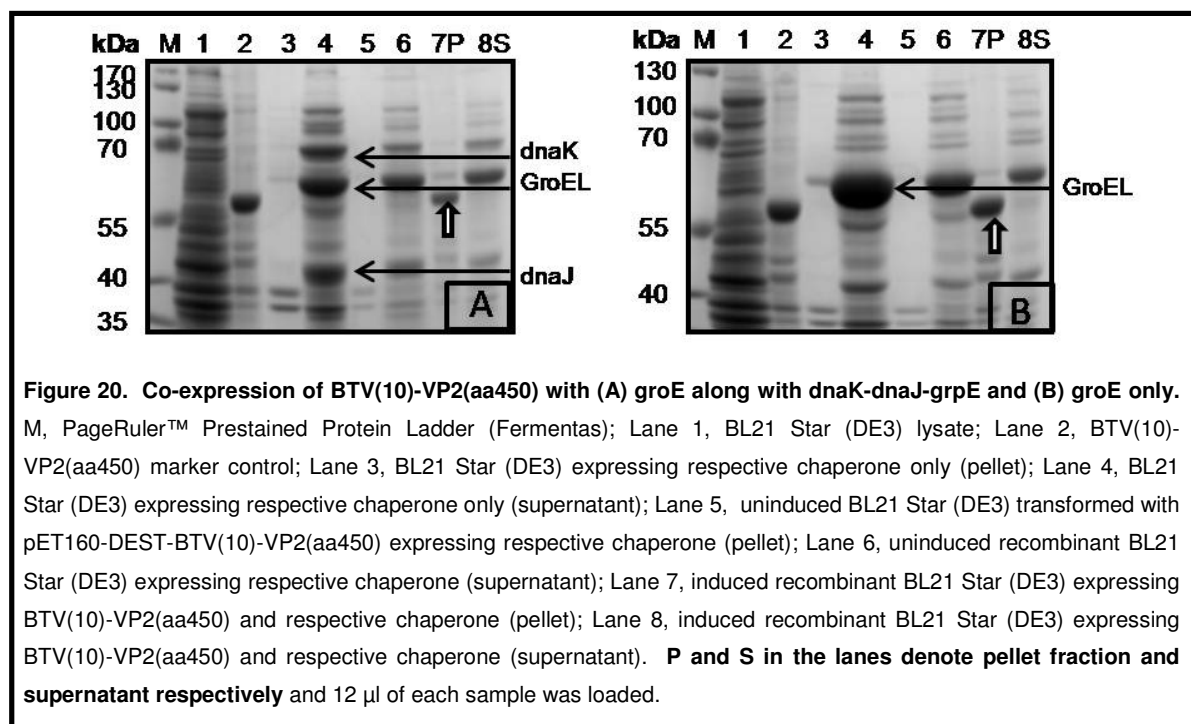
3.4.4.2. Co-expression of BTV(10)-VP2(aa450) proteins with chaperones groE and dnaK-dnaJ-GrpE

The groEL-groES (groE) chaperone system can only assist in folding of polypeptide chains that are < 60 kDa. Therefore it was only co-expressed with BTV(10)-VP2(aa450) which has a theoretical molecular weight of 55 kDa. The exposed hydrophobic regions of newly synthesized polypeptide chains bind to the hydrophobic walls of the central cavity of the GroEL chaperone. The polypeptide chain is only released due to a change in the hydrophilicity of the wall cavity of the GroEL chaperone when the GroEL binds a GroES chaperone. The polypeptide chain is then free to fold to its tertiary conformation within the hydrophilic cavity of the GroEL chaperone (Xu and Sigler 1998). In the following set of experiments, the BTV(10)-VP2(aa450) protein was expressed with either the groE chaperone only or with the groE chaperone together with the dnaK-dnaJ-GrpE chaperones used in Section 3.4.4.1 above.

A single bacterial colony containing the appropriate chaperone plasmid (Takara) and recombinant pET160-DEST was cultured in LB-broth containing the appropriate inducer (4.0 mg/ml L-arabinose and/or 10 ng/μl tetracycline) to the chaperone promoter. The bacterial culture was grown at 30°C until an OD₆₀₀ 0.4 – 0.6. Recombinant protein expression was induced with 1.0 mM IPTG and continued for 4 hours at 30°C. BL21 Star (DE3) cells expressing the chaperones were used as a control to monitor the chaperone expression. Non-transformed BL21 Star (DE3) cells, uninduced recombinant BL21 cells and BL21 lysate containing expressed BTV(10)-VP2(aa450) protein were also included as controls. After recombinant protein expression, the cells were collected, lysed and fractionated by high speed centrifugation into the soluble and insoluble fraction. Approximately 12 μl of each samples was analyzed with 12% SDS-PAGE stained with Coomassie blue G-250 (Figure 20).

With reference to Figure 20, cells expressing putative chaperones only (Lane 4) contained proteins corresponding to the theoretical molecular weight of dnaK, dnaJ and groEL (70, 40 and 57 kDa respectively). These proteins were not expressed in the non-recombinant BL21 cells (Lane 1). The chaperones GroES and GrpE with a molecular weight of 10 and 20 kDa respectively are too small to be detected on the polyacrylamide gels above. However the expression of groES and GrpE were assumed since both chaperones are encoded by the same vector as the dnaK, dnaJ and groEL. The chaperone proteins were expressed at high levels and were found in the supernatant (Lane 4). This is indicative of the presence of native chaperonin structures. A protein of similar molecular weight to that of the control BTV(10)-VP2(aa450) marker protein (Lane 2) was observed in the induced culture pellet (indicated by arrows in Lane 7P). Therefore the co-expression of the BTV(10)-VP2(aa450) protein with either the GroE chaperones only or the GroE chaperones in combination

with the previously successful dnaK-dnaJ-grpE chaperones (Section 3.4.4.1 above) did not produce soluble BTV(10)-VP2(aa450) proteins.



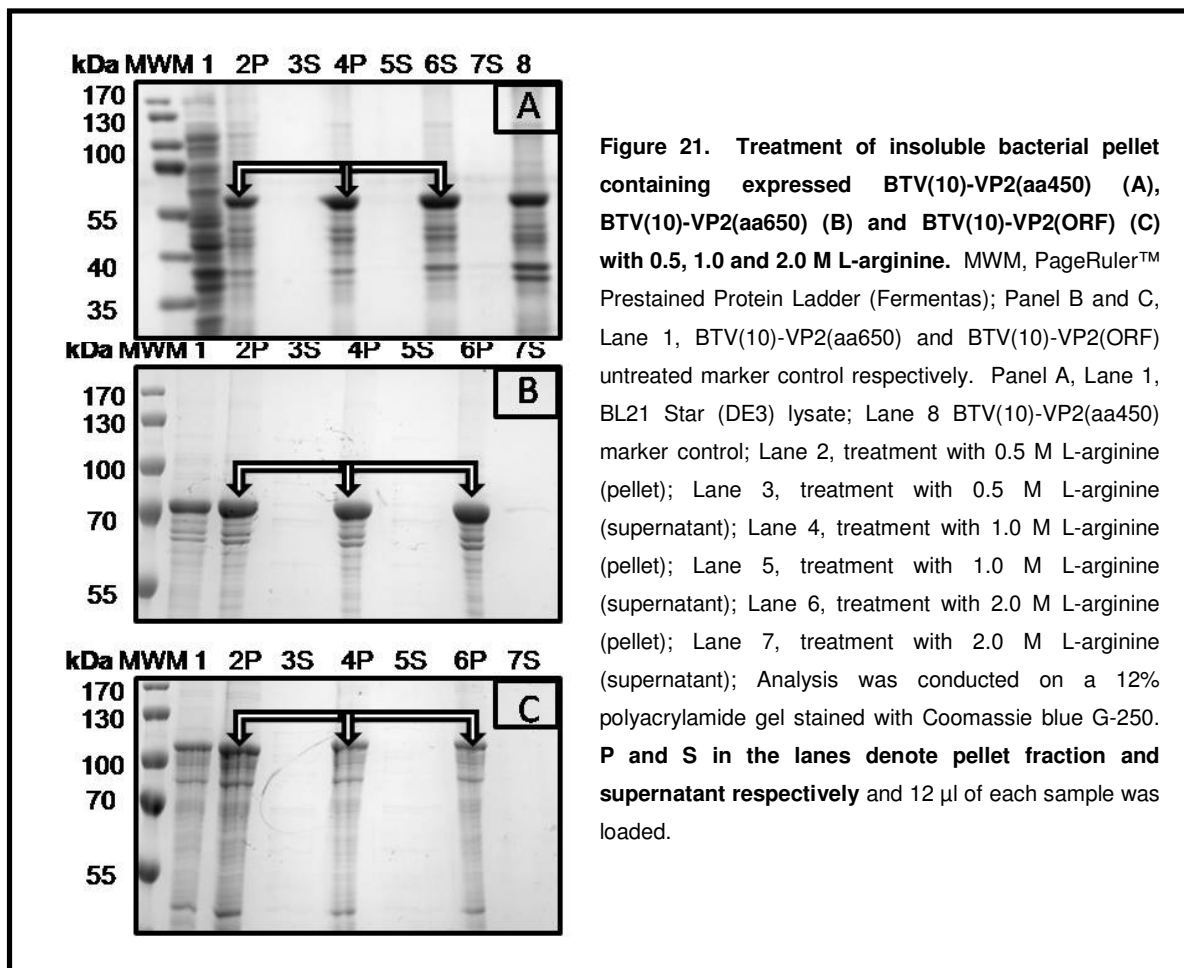
Thus far, only co-expression of molecular chaperones, dnaK-dnaJ-GrpE was successful at producing some soluble recombinant BTV(10)-VP2 proteins. Therefore in next set of experiments the aim was to try to recover soluble full-length BTV(10)-VP2 and truncated proteins from the inclusion bodies. This was done using L-arginine to reverse aggregation of the recombinant proteins.

3.4.5. Arginine treatment of inclusion bodies containing expressed BTV(10)-VP2 recombinant proteins

Arginine treatment of the pellet from the pilot study was based on a previous study conducted by Tsumoto, et al. in 2003. The arginine treatment of aggregated GFP released putative native and/or partially folded (near-native) fluorescent proteins (Tsumoto, Umetsu et al. 2003). In the following experiment, L-arginine was used to firstly disaggregate the inclusion body and secondly to prevent aggregation from taking place subsequent to reversing aggregation of the recombinant proteins.

The inclusion body i.e. the pellet from a 10 ml recombinant culture induced with 1.0 mM IPTG and grown at 37°C for 4 hours was treated with varying concentrations of L-arginine in STE (0.5 M, 1.0 M and 2.0 M). The treated pellet was incubated at room temperature for 1 hour and then separated into soluble and insoluble fractions with high speed centrifugation. The fractions and an untreated

pellet fraction containing the target protein as a marker control were analyzed with SDS-PAGE (Figure 21).



Arginine treatment of the bacterial inclusion body containing expressed recombinant BTV(10)-VP2 proteins did not reverse aggregation since the recombinant proteins remained in the insoluble fraction after high speed centrifugation (Figure 21, Lane 2P, 4P and 6P). It is possible that high speed centrifugation of the bacterial lysate from the pilot study caused an increased compaction of the already highly aggregated inclusion body thereby preventing the action of L-arginine, even at high concentrations.

Since the treatment of the inclusion body with L-arginine was unsuccessful at reversing aggregation, the cell lysates containing the recombinant BTV(10)-VP2 proteins were treated with L-arginine with the aim of reversing aggregation of the recombinant proteins.

3.4.6. Arginine treatment of bacterial lysate containing the recombinant BTV(10)-VP2 proteins

Since the arginine treatment of the bacterial **pellets** containing recombinant BTV-VP2 proteins did not reverse aggregation of the inclusion body and release putative soluble proteins (above), in the current experiment, the bacterial **lysate** containing the recombinant proteins were treated with 1.0 M L-arginine and incubated at room temperature for 1 hour. The aim here was to use L-arginine to reverse aggregation of the recombinant BTV(10)-VP2 proteins present in the host cell lysate to release putative soluble recombinant protein. As a control reaction, the bacterial lysate of each recombinant protein was also treated with 1% sarkosyl and incubated for 1 hour at room temperature. Sarkosyl is a mild detergent which should denature proteins that are not highly aggregated. The arginine and sarkosyl treated lysates were fractionated by high speed centrifugation into the soluble and insoluble fractions. The soluble and insoluble fractions were analyzed on a 12% SDS polyacrylamide gel (Figure 22). Untreated lysates containing the appropriate BTV-VP2 recombinant proteins were used as a marker control.

SDS-PAGE analysis showed that the recombinant BTV(10)-VP2 proteins remained in the insoluble fraction after treatment of the bacterial lysate with 1.0 M arginine (Figure 22, A1, B1 and C1). The arginine treatment therefore did not reverse aggregation of the recombinant protein within the lysate to release putative soluble proteins. Further, the sarkosyl treatment did not fully denature the recombinant proteins since these proteins were predominantly present within the pellet after treatment (indicated by arrows A2, B2 and C2 respectively in Figure 22). This is in accordance with previous findings where it was shown that sarkosyl does not completely denature highly aggregated proteins. Complete denaturation requires strong denaturants such as urea or guanidine-HCl (Dr Quinton. Meyer, personal communication, University of Pretoria). Since the sarkosyl, a mild chaotrope, was ineffective at complete denaturation; it was expected that L-arginine would also be ineffective at reversing aggregation of the recombinant proteins.

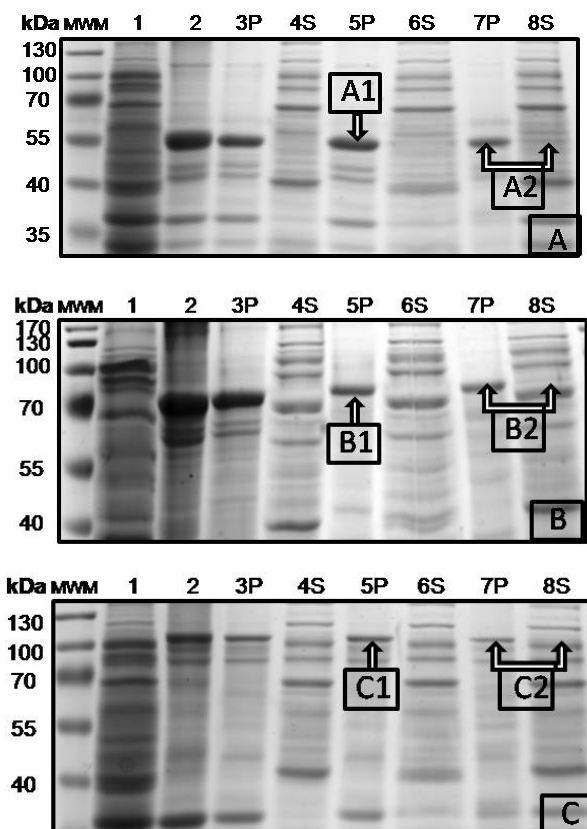


Figure 22. SDS-polyacrylamide gel analysis of fractionated, arginine and sarkosyl treated bacterial lysates containing expressed BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C). MWM, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, BL21 Star (DE3) lysate; Lane 2, Panel A: BTV(10)-VP2(aa450), Panel B: BTV(10)-VP2(aa650) and Panel C: BTV(10)-VP2(ORF) marker control; Lane 3, untreated pellet fraction; Lane 4, untreated supernatant fraction; Lane 5, treatment of BTV(10)-VP2(aa450) (A1), BTV(10)-VP2(aa650) (B1) and BTV(10)-VP2(ORF) (C1) lysate with 1 M L-arginine (pellet); Lane 6, treatment of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) lysate with 1 M L-arginine (supernatant); Lane 7, treatment of BTV(10)-VP2(aa450) (A1), BTV(10)-VP2(aa650) (B1) and BTV(10)-VP2(ORF) (C2) lysate with 1% sarkosyl (pellet); Lane 8, treatment of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) lysate with 1% sarkosyl (supernatant). **P and S in the lanes denote pellet fraction and supernatant respectively** and 12 µl of each sample was loaded.

The soluble and insoluble proteins of the untreated and arginine treated bacterial lysates (above) were separated using high speed centrifugation. In the next set of experiments, the BTV(10)-VP2 proteins were separated with sucrose gradient ultracentrifugation.

3.4.7. Sucrose gradient analysis of bacterial lysate containing the recombinant BTV(10)-VP2 proteins

The analysis of the bacterial lysate containing the recombinant BTV-VP2 proteins on a sucrose gradient has not been previously described. The problem with high speed centrifugation is that it is

possible for soluble proteins to be “pulled” with the aggregated proteins to the insoluble fraction, a phenomenon referred to as mass action (Horn and Jackson 1972). The real advantage with the sucrose gradient is that it gives one a more accurate indication of the soluble, partially aggregated and insoluble proteins. These proteins are found at the top (fractions 7 – 10), middle (fractions 4 – 6) and lower sucrose fractions (fractions 1 – 3 and particulate) respectively after ultracentrifugation. The advantage of the sucrose gradient technique is that one may separate the soluble component of the recombinant proteins from the particulate which may then be used as an antigen. This is dependent on a significant yield (unpublished data, University of Pretoria).

Under this section, the cell lysates from the pilot study and the cell lysates treated with L-arginine were assessed on a sucrose gradient by ultracentrifugation (current chapter, section 2.12.1.2). The aim here was to determine the sedimentation pattern and assess the aggregation status of the recombinant proteins.

3.4.7.1. *Sucrose gradient analysis of bacterial lysates containing expressed recombinant BTV(10)-VP2 proteins*

The sedimentation pattern of the recombinant BTV(10)-VP2 proteins from the bacterial cell lysate through a sucrose gradient was analyzed with SDS-PAGE. The aim here was to determine the status of aggregation of the expressed recombinant BTV(10)-VP2 proteins. A single recombinant BL21 Star (DE3) was inoculated into 8 ml LB broth containing appropriate antibiotics and grown to an OD₆₀₀ 0.4 – 0.6 at 37°C. Expression was induced with 1 mM IPTG and continued for 4 hours at 37°C. Thereafter the cells were collected and lysed with 800 µl cell lysis buffer (pH 7.8). The resulting lysate was layered on a 50 – 70% discontinuous sucrose gradient and centrifuged in an ultracentrifuge at 30 000 rpm for 18 hours at 4°C. Approximately 500 µl per fraction was collected from the bottom to the top of the gradient and labeled 1 to 10 while the particulate fraction was labeled P. Approximately 20 µl of each fraction and pellet collected were analyzed on a 12% polyacrylamide gel stained with Coomassie blue G-250 (Figure 23). Non-transformed BL21 Star (DE3) cells were used as a negative control while recombinant BL21 Star (DE3) cell lysate expressing the appropriate recombinant BTV-VP2 protein was used as a positive control.

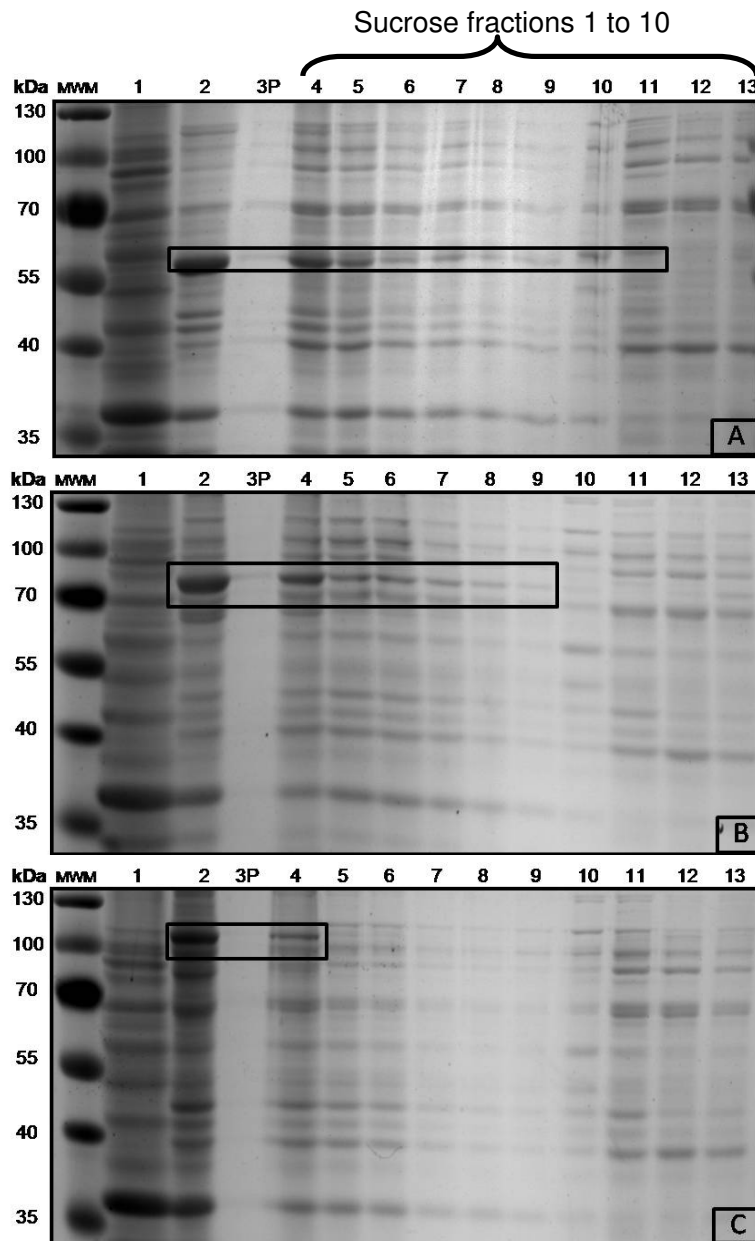


Figure 23. SDS-polyacrylamide gel analysis of sedimentation pattern of bacterial lysate containing expressed BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) on a 50 – 70% discontinuous sucrose gradient. M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, non-transformed BL21 Star (DE3) cell lysate; Lane 2, induced recombinant BL21 Star (DE3) lysate with recombinant control protein. Resuspended pellet after ultracentrifugation is indicated in Lane labeled 3P while Lane 4 – 13 represents harvested sucrose gradient fractions 1 to 10 respectively. Boxes show location of where the recombinant proteins lie. **P in the lane denotes particulate fraction** and 20 µl of each sample was loaded.

In Figure 23, the recombinant BTV(10)-VP2 proteins marker control (Lane 2) was used to show the position of the recombinant proteins which were not present in the non-transformed BL21 Star (DE3)

(Lane 1). It should be noted that the yield of the recombinant proteins from the sucrose gradient cannot be compared to the marker control protein since the culture volume and amount loaded per gel was not the same (20 µl for the sucrose fractions and 12 µl for the marker control). BTV(10)-VP2(aa450) was found in sucrose fractions 1 to 8, BTV(10)-VP2(aa650) in sucrose fractions 1 to 6 while BTV(10)-VP2(ORF) in sucrose fractions 1 (indicated by boxes). The sedimentation of the BTV(10)-VP2(aa450) proteins to the sucrose fractions 7 and 8 is either indicative of putative soluble proteins. This was not previously observed in the soluble fraction of the untreated lysate obtained after high speed centrifugation. The presence of the BTV(10)-VP2(aa450) proteins in sucrose fractions 7 and 8 could alternatively be an overlap of the host BL21 native proteins which have a similar molecular weight to this protein. Therefore verification by immunoblot analysis is necessary (this was not possible due to the limited supply of anti-BTV IgY antibodies). The sucrose fraction 1 contained more of the recombinant proteins than the particulate fraction. This was probably due to the high density of the lower sucrose fraction preventing the aggregated proteins from passing through.

3.4.7.2. Sucrose gradient analysis of arginine treated bacterial lysates containing recombinant BTV(10)-VP2 proteins

The sedimentation pattern of arginine-treated recombinant BTV(10)-VP2 proteins present in the bacterial cell lysate was analyzed with SDS-PAGE. The aim here was to determine if L-arginine would reverse aggregation of the inclusion bodies. The protocol here was essentially the same as above expect that following protein expression, the cells were lysed with 400 µl cell lysis buffer and treated with 1 M L-arginine. The suspension was incubated at room temperature for 1 hour and thereafter also layered on a 50 – 70% discontinuous sucrose gradient. The above mentioned controls were once again used and also approximately 20 µl of each sample was analyzed with SDS-PAGE stained with Coomassie G-250 (Figure 24).

The sedimentation pattern of the treated lysate was essentially the same as the untreated bacterial lysate containing the recombinant proteins (Figure 24). BTV(10)-VP2(aa450) was found in sucrose fractions 1 to 8, BTV(10)-VP2(aa650) in sucrose fractions 1 to 6 while BTV(10)-VP2(ORF) in sucrose fractions 1 only. Since there was a correlation of the results obtained for untreated and arginine treated lysates on a sucrose gradient, L-arginine therefore did not disaggregate the inclusion bodies.

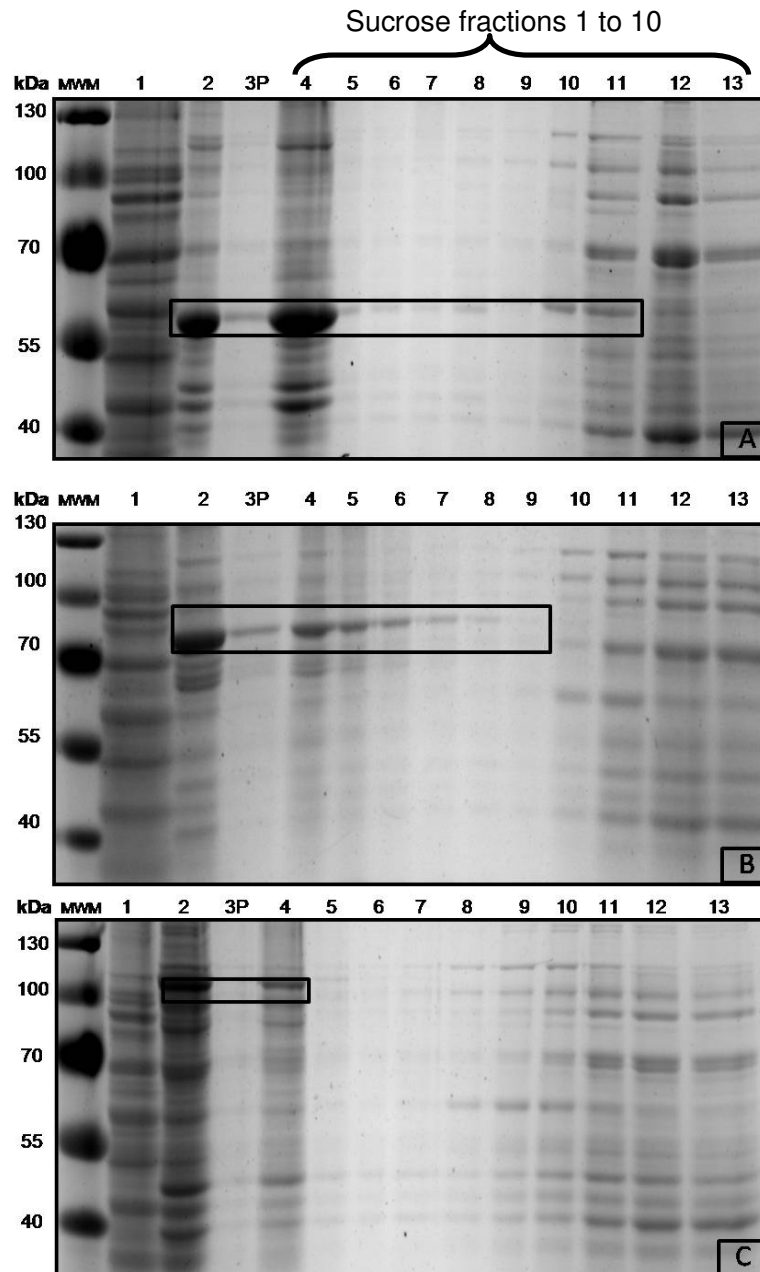


Figure 24. Sedimentation pattern of arginine treated bacterial lysate containing expressed BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) on a 50 – 70% discontinuous sucrose gradient. M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, non-transformed BL21 Star (DE3) cell lysate; Lane 2, induced recombinant BL21 Star (DE3) lysate with recombinant control protein. Resuspended pellet after ultracentrifugation is indicated in Lane labeled 3P while Lane 4 – 13 represents harvested sucrose gradient fractions 1 to 10 respectively. Boxes show location of where the recombinant proteins lies and analysis was conducted on SDS-polyacrylamide gel stained with Coomassie blue G-250 **P in the lane denotes particulate fraction** and 12 µl of each sample was loaded.

4. DISCUSSION

The research reported in the current chapter is focused on the use of prokaryotic expression to produce soluble full-length BTV(10)-VP2 and truncated versions of this protein which are predicted from literature to contain BTV neutralizing epitopes (White and Eaton 1990; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; DeMaula, Bonneau et al. 2000; Bhattacharya, Noad et al. 2007; Schwartz-Cornil, Mertens et al. 2008). Previous attempts at using BTV-VP2 as a subunit vaccine included using the BTV-VP2 protein by itself or as part of VLPs (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). The VLPs are an attractive vaccine strategy since the particles resemble the authentic BTV, however it is difficult to produce large quantities and the stability decreases during long-term storage (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). The vaccine potential of BTV-VP2 by itself has been demonstrated (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). When looking at a single protein as a subunit vaccine, solubility is an important consideration since the proteins needs to fold in an ordered tertiary structure to expose the neutralizing epitopes as found on the intact viral particle (Liljeqvist and Stahl 1999). Large antigen usually aggregate when expressed *in vitro* due to non-native interactions between exposed hydrophobic regions. The immune response toward aggregated proteins is usually weak. The problem of insolubility can be dealt with by the expression of truncated versions of a particular antigen which contains the neutralizing epitopes but lacks hydrophobic regions. The success of this strategy is dependent on a known atomic structure of a particular antigen derived from X-ray crystallography as well as prior knowledge of the positioning of the neutralizing epitopes (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009). To date there is no predictive software to successfully model the tertiary structure of a protein. The lack of an atomic structure of BTV-VP2 complicates the design of a truncated vaccine antigen. BLAST analysis of the BTV(10)-VP2 amino acid sequence with sequences for which the tertiary structure is known revealed a 0% identity (data not shown). An extensive literature search has revealed a general consensus regarding the position of the neutralizing epitopes lying within the first 450 amino acids of the VP2 protein (White and Eaton 1990; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; DeMaula, Bonneau et al. 2000; Bhattacharya, Noad et al. 2007; Schwartz-Cornil, Mertens et al. 2008). In the current study, an *in silico* analysis of BTV(10)-VP2 primary amino acid sequence was carried out to characterize the protein in terms of hydrophobicity distribution in relation to the position of the neutralizing epitopes. The analysis revealed an overlap between the N-terminal of BTV(10)-VP2 (first section of BTV(10)-VP2) which is associated with viral neutralization and regions that displayed an overall lower hydrophobicity (Figure 7). From the results in Figure 7, two truncated BTV(10)-VP2 proteins were designed which lacked C-terminal hydrophobic regions i.e. BTV(10)-

VP2(aa450) (amino acid 1 to 450) and BTV(10)-VP2(aa650) (amino acid 1 to 650). The smallest truncated protein contained only the previously reported neutralizing epitopes while the larger truncated proteins contained a greater portion of VP2 backbone in addition to the reported neutralizing epitopes (Figure 8). The tertiary conformation of these truncated proteins relative to the full-length BTV-VP2 protein is unknown. Therefore the larger truncated protein was designed since it might provide better options for correct tertiary folding. Although the larger truncated protein was more hydrophobic than the BTV(10)-VP2(aa450) protein, its hydrophobicity was still essentially lower than the remainder of the full-length BTV-VP2 protein.

In this chapter the solubility analysis was conducted on the full-length BTV-VP2 and the truncated BTV(10)-VP2 proteins which were expressed in a prokaryotic expression system. One of the main advantages of bacterial expression is the low cost associated with rapid and high productivity of heterologous proteins (Georgiou and Valax 1996). Currently there are no reported studies describing the prokaryotic expression of these recombinant BTV-VP2 proteins. It might also be necessary to mention that the lack of a post-translational modification pathway in the prokaryotic expression system should not be of a disadvantage since BTV-VP2 is a non-glycosylated protein (Roy 2008).

A pilot expression of full-length BTV(10)-VP2 and truncated versions thereof was carried out in *E. coli* BL21 Star (DE3) cells using the recombinant pET160-DEST expression vector. In addition to confirming expression and identity of 55 kDa BTV(10)-VP2(aa450), 70kDa BTV(10)-VP2(aa650) and 114 kDa BTV(10)-VP2(ORF) with SDS-PAGE and immunoblot analysis, the pilot expression study also revealed that all of the expressed proteins were insoluble since they could only be found in the insoluble fraction after high speed centrifugation (Figure 12 and Figure 13). The removal of the hydrophobic regions of BTV(10)-VP2 at the C-terminal end therefore did not affect solubility. The recombinant proteins were however being synthesized at extremely high levels with the order of magnitude being BTV(10)-VP2(aa450) > BTV(10)-VP2(aa650) > BTV(10)-VP2(ORF). This is due to the difference in length of the transcripts with larger proteins requiring more time for transcription and translation (Studier and Moffatt 1986).

In the bacterial system, the presence of the recombinant proteins in the insoluble fraction is indicative of an inclusion body. This is a common occurrence during cytoplasmic over-expression in prokaryotic expression systems. The inclusion body is a highly aggregated entity comprising predominantly of recombinant over-expressed proteins (Sahdev, Khattar et al. 2008). In terms of the current study, when the host T7 RNA polymerase is fully induced by IPTG, the majority of the host cell resources are directed toward the target gene expression. The T7 polymerase is usually five times faster than the *E. coli* RNA polymerase and therefore targets gene mRNA synthesis and

accumulation usually reaches levels comparable to the ribosomal RNAs in untransformed cells. It has been suggested that the mRNA produced by the T7 RNA polymerase is also protected from intracellular exonucleolytic degradation which in effect increases the half-life of the transcripts (Studier and Moffatt 1986). The abundance of transcripts produced can rapidly saturate the host cells translation machinery while the polypeptide chains can overburden the host's protein folding chaperones and folding catalysts. This accounts for the high level expression. Additionally the lack of a transport secretion signal tag on the pET160-DEST vector means that the expressed proteins are destined for cytoplasmic localization. This results in a variety of cytoplasmic distributed proteins with folding characteristics that range from misfolded, partially-folded (near native) and putative soluble proteins (Georgiou and Valax 1996; Tsumoto, Umetsu et al. 2003). Due to the exposure of hydrophobic patches, the misfolded and partially-folded proteins may interact and be sequestered into a refractile aggregated network termed inclusion body (Ventura and Villaverde 2006).

A general strategy for improving solubility and circumventing aggregation is to optimize protein expression by changing expression conditions that directly impact on the level of recombinant proteins expressed (Moghaddam and Bicknell 1992; Makrides 1996). The expression conditions that were under investigation in the current study included induction conditions, fermentation temperature and harvest times (Figure 14, Figure 15 and Figure 16). This part of the study revealed a correlation between the above mentioned expression conditions and the quantity of recombinant protein being synthesized. High levels of inducer, high fermentation temperature and late harvest times resulted in an increase in the quantity of proteins being synthesized while lower inducer concentrations, fermentation temperature and early harvest times resulted in small quantities or lack of recombinant proteins being synthesized. The study however did not show a relationship between such expression conditions and the synthesis of soluble recombinant proteins since the recombinant proteins remained in the insoluble fraction after high speed centrifugation (Figure 14, Figure 15 and Figure 16) (discussed below).

Tsumoto, et al. in 2003 showed that the arginine treatment of aggregated GFP released putative native and/or partially folded (near-native) fluorescent proteins (Tsumoto, Umetsu et al. 2003). Arakawa and Tsumoto also showed in 2003 that L-arginine played a role in the reversing aggregation (Arakawa and Tsumoto 2003). In this study the arginine treatment of the bacterial inclusion body or bacterial lysate containing the recombinant BTV(10)-VP2 proteins did not solubilize the proteins. The recombinant proteins remained insoluble after high speed centrifugation (Figure 21 and Figure 22 respectively). Various studies have demonstrated that disaggregation of inclusion bodies are difficult and only achieved through the use of harsh denaturants such as urea (at 8 M) or guanidine-HCl (Dr Quinton Meyer, personal communication, University of Pretoria). This might explain why the anionic detergent sarkosyl was unable to denature the inclusion body (Figure 22).

Therefore if sarkosyl was unable to perform its function, it is unlikely that L-arginine would be effective in reversing aggregation. The increase of compaction of the bacterial inclusion body i.e. the pellet fraction during high speed centrifugation might also explain the ineffectiveness of the arginine treatment of the pellet to reverse aggregation even under the increased concentrations of L-arginine. With regard to the sarkosyl and arginine treatment of the bacterial lysate, it was possible that the treatment might partially disaggregate the inclusion body. However, during high speed centrifugation the proteins once again move by mass action with the remainder of the aggregated protein (Horn and Jackson 1972).

Since the various optimizations described above were unsuccessful in reversing aggregation of the expressed BTV(10)-VP2 and truncated proteins it is important to scrutinize the constant factors of these experiments i.e. the recombinant proteins itself and the expression system. For instance, the full-length BTV-VP2 protein is 110 kDa. During *in vitro* synthesis, longer polypeptides tend to interact non-natively as they leave the ribosomal unit. Intermolecular hydrophobic interactions may arise between exposed hydrophobic patches resulting in protein aggregation (Frydman 2001; Hartl and Hayer-Hartl 2002).

In addition, the cytosol of the bacterial host is a highly reducing environment and is not conducive for efficient disulfide bond formation which requires the oxidation of the thiol groups of cysteine residues (Aslund and Beckwith 1999; Woycechowsky and Raines 2000). This reaction is necessary for the productive folding pathway of disulfide containing proteins and is of particular importance and relevance when considering that the recombinant BTV(10)-VP2 proteins contains 6, 8 and 15 cysteine residues respectively. In addition to this the Lumio™ tag contains 2 cysteine residues. There is no reliable bioinformatics software that can accurately predict possible disulfide bonds existing within the BTV-VP2 derived proteins nor is there any evidence thereof. However it is highly probable that there exists the minimal number of disulfide bonds due to the above mentioned number of cysteine residues. The absence of putative disulfide bonding may lead to the instability of the recombinant proteins and result in partially folded proteins which promote aggregation. This may account for the formation of the inclusion bodies even at low concentrations of inducer, low fermentation temperatures and early harvest times (Figure 14, Figure 15 and Figure 16). The opposite can also be true since in 1990, Miele et al. demonstrated formation of two interchain disulfide bonds within the bacterial cytoplasm (Miele, Cordella-Miele et al. 1990). Due to the fact that disulfide bonds may be formed in the cytoplasm, it is possible that non-native disulfide bonds within the BTV(10)-VP2 recombinant proteins were formed, thus producing misfolded proteins and/or partially folded proteins. This suggests that the inclusion bodies produced during the above

experiments contained highly aggregated proteins due to intermolecular interactions between the misfolded and/or partially-folded recombinant polypeptide chains.

The next set of experiments involved the co-expression of molecular chaperones dnaK, dnaJ and GrpE with the recombinant proteins. Through a series of binding and release cycles, these chaperones assist with folding of larger multidomain proteins (> 60 kDa). The dnaK chaperones bind exposed hydrophobic regions of the polypeptide chain which prevents premature intramolecular misfolding. When released, the peptide chain is free to fold to its native conformation (Bukau and Horwich 1998). In this project, the aim was to over-express these 'helper molecules' to assist in the complete folding of the expressed recombinant BTV(10)-VP2 proteins. Interestingly the co-expression of chaperones dnaK-dnaJ-GrpE with the recombinant proteins resulted in a soluble fraction of recombinant BTV(10)-VP2 proteins (Figure 18). This means that the chaperones dnaK-dnaJ-GrpE played a role in the complete folding of a percentage of the recombinant proteins. With regard to the BTV(10)-VP2(aa450) protein, approximately 50% of the expressed recombinant protein was present in the soluble fraction while approximately 20% of the BTV(10)-VP2(aa650) protein and full-length BTV(10)-V2 was present in the soluble fraction. To emphasize the impact of such a find it is important to note that this is the first study demonstrating the synthesis of soluble recombinant BTV-VP2 proteins in a prokaryotic system and that the soluble proteins present on the polyacrylamide gel is only 10 µl from a 10 ml bacterial culture after four hours of protein expression. It should be mentioned that arginine treatment of the bacterial lysate co-expressing chaperones dnaK-dnaJ-GrpE with the recombinant BTV(10)-VP2 proteins did not increase the fraction of soluble proteins (data not shown).

In 1990 Miele et al. demonstrated the correct folding of a 70 amino acid dimeric uteroglobin in *E. coli*. According to crystallographic studies, in addition to the two disulfide interactions, there are several other stereo-specific interactions such as Van der Waals and Hydrogen bonding within the uteroglobin quaternary structure. The researchers hypothesize that during the folding process, non-covalent interactions stabilize the protein intermediate thereby bringing the cysteine residues into the appropriate position that favors only "correct" disulfide bond formation resulting in the dimers. By use of this model, one can postulate that the bind-and-release mechanism of dnaK-dnaJ-GrpE with the BTV-VP2 derived proteins promotes primary native non-covalent interactions within the recombinant proteins. This may be adequate to produce correctly folded BTV(10)-VP2 derived proteins if there are no disulfide bonds present in the recombinant BTV(10)-VP2 proteins in the first place. Alternatively it could bring the cysteine residues in close proximity to allow for native disulfide bridge formation resulting in native conformation of the recombinant proteins.

Another hypothesis supporting the synthesis of soluble recombinant BTV(10)-VP2 proteins when these recombinant proteins are co-expressed with dnaJ-dnaK-GrpE is the formations of disulfide bonds within the oxidizing environment of the endoplasmic reticulum (ER) (Tu, Ho-Schleyer et al. 2000). The hsp70 or equivalent dnaK in *E. coli* also binds newly synthesized polypeptides which are in the process of being translocated from the cytoplasm to the ER. The ER is rich in chaperones including hsp70, immunoglobulin heavy chain binding protein (BiP) and enzymes such as protein disulfide isomerase which catalyze disulfide bond formation (Fink 1999). The co-expression of the dnaK-dnaJ-GrpE with the BTV-VP2 derived proteins reduced the expression of the recombinant proteins since the translational machinery is now shared between the various over-expressed proteins. At this low level of recombinant protein synthesis, the chaperones, dnaK-dnaJ-GrpE, were able to circumvent misfolding by binding the polypeptide chains which were translocated to the ER. In the ER, where the dnaK, other chaperones and enzymes which promote effective disulfide bond formation are highly concentrated, were then able to efficiently fold the recombinant proteins (Fink 1999).

One may also argue that during low level expression of recombinant proteins with the chaperones, dnaK-dnaJ-GrpE more time is allocated for the complete folding of the synthesized recombinant proteins. This however contradicts the results found during the optimization of the expression conditions when inducer concentration, fermentation temperature and harvesting time were reduced and no soluble recombinant BTV(10)-VP2 proteins were detected.

The next step was the purification of the above soluble fraction of recombinant BTV(10)-VP2 proteins. Non-denaturing purification of these soluble recombinant proteins was attempted by virtue of their N-terminal polyhistidine tag using nickel chromatography (Figure 19). Although a small fraction of the BTV(10)-VP2(aa450) protein eluted in the final elution step, the bulk of the recombinant BTV(10)-VP2 proteins eluted with the initial flow-through or wash 1 and 2. This is further verification that the recombinant proteins are soluble since they did pass through the nickel columns (insoluble aggregates cannot pass through the columns). The inability to bind to the nickel-containing resin is probably due to the tertiary conformation of the recombinant proteins. According to the manufacturers a single histidine residue from the polyhistidine tag binds to a single coordination site of the nickel ion immobilized on a silica bead (Protino® Ni-TED manual). Based on the theory behind the purification technique and the fact that this was a non-denaturing purification protocol, it is possible that the tertiary structure of the recombinant proteins buries the histidine tag thus preventing exposure of a single N-terminal histidine residue. The inability to purify these proteins is not of a disadvantage since various studies have demonstrated the potential of Gram negative outer-membrane component lipopolysaccharide (LPS) as an effective adjuvant (Chedid,

Audibert et al. 1976; Dimov, Ivanovska et al. 1992; Alving 1993; Trott, Hellestad et al. 2008). Time constraints prohibited further investigation and this was beyond the scope of this project.

Co-expression studies were also conducted with the chaperones GroEL-GroES (GroE). Since the GroE chaperones can only assist in the folding of polypeptide chains smaller than 60 kDa, it was only co-expressed with the BTV(10)-VP2(aa450) protein (55 kDa). Exposed hydrophobic regions of polypeptide chains bind to the hydrophobic walls of the central cavity of the GroEL chaperone. The polypeptide chain is released due to a change in the hydrophilicity of the wall cavity of the GroEL chaperone upon the binding of the GroES chaperone. The polypeptide is therefore free to fold to native state within the hydrophilic cavity of the GroEL chaperone (Xu and Sigler 1998). Interestingly the co-expression of the BTV(10)-VP2(aa450) protein with only the GroE chaperone and GroE with the DnaK-DnaJ-GrpE chaperone does not produce soluble protein (Figure 20). It is possible that the GroE chaperones could not assist in complete folding of BTV(10)-VP2(aa450) as the recombinant proteins misfolded and/or aggregated prior to coming into close proximity with the cytosolic GroE chaperones. This means that the proteins exceeded the allowed molecular weight of GroE. The expression of the recombinant BTV-VP2 proteins in heat-shocked and salt-stressed host BL21 cells also did not produce soluble proteins (Figure 17). The choice of the correct chaperone for protein folding is very important. According to Hoffmann and Rinas, the incorrect chaperone will not result in successful protein folding and can affect the proteins stability. (Hoffmann and Rinas 2004). This means that the native chaperones over-expressed during the heat and salt stress may not necessarily be involved in the folding of the foreign recombinant proteins but probably caters to the host native proteins. Therefore no soluble recombinant BTV(10)-VP2 proteins were detected. The decrease in the quantity of recombinant proteins as compared to the initial pilot study (Figure 17 and Figure 12 respectively) was probably due to the increase in host native proteases expression during heat shock which breakdown aggregated and misfolded proteins (Christoph, Beil et al. 2000)

Up until now the standard fractionation protocol involved high speed centrifugation. The concept of mass action is very important when using this form of centrifugation since during high level expression of a particular protein, putative soluble proteins can be “pulled” to the particulate fraction by an “umbrella effect” during the sedimentation of the aggregation to the bottom of the vessel (Horn and Jackson 1972). Sucrose gradient ultracentrifugation is advantageous over high speed centrifugation as one is able to separate soluble (sucrose gradient 7 -10), partially aggregated (1 – 6) and highly aggregated proteins (particulate fraction). The sucrose gradient analysis of the untreated bacterial lysates containing the recombinant proteins was essentially the same as that of the lysate treated with L-arginine. The implication here is that L-arginine did not affect the inclusion body. The BTV(10)-VP2(aa450) proteins found in sucrose fractions 7 and 8 suggest that this could

be soluble proteins being expressed. However this could also be the host BL21 native proteins which are in a similar molecular weight size range as the BTV(10)-VP2(aa450) protein. Therefore further verification of protein identity by immunoblot is required. This was not possible in this study due to the limited supply of anti-BTV(10) antibodies. The BTV(10)-VP2(aa650) and full-length VP2 proteins were only found sucrose fractions 1 to 6 and fraction 1 respectively. This suggests that these proteins were highly aggregated.

CHAPTER 3: BACULOVIRUS-Sf9 EXPRESSION OF FULL-LENGTH BTV-VP2 AND TRUNCATED VERSIONS OF THIS PROTEIN

1. INTRODUCTION

The baculovirus-insect cell expression system is a valuable tool for high level synthesis of foreign proteins (Ailor and Betenbaugh 1999). This system has the marked advantage over the prokaryote expression system as it is able to mimic to a degree the post-translational processing found in mammalian cells (Jarvis and Finn 1996; Jarvis, Howe et al. 2001; Kost, Condreay et al. 2005). The downside of this system however is shut-down of the host native protein synthesis by the strong baculovirus polyhedron promoter. This in-turn means that there is a limitation of protein factors involved in the secretory pathway following recombinant baculovirus infection. The available factors which are imperative in folding and assembly become overburden leading to misfolded and partially folded protein intermediates aggregating (Ailor and Betenbaugh 1999; Kavar and Jarvis 2001). Protein aggregation can be reversed by the use of disaggregating agents such as L-arginine or a denaturation and refolding strategy. It is hypothesized that the positive charge of L-arginine surround the proteins causing a repulsive force which in turn disaggregates and prevents further aggregation while solubilization and refolding consists of the complete denaturation of the recombinant proteins followed by the refolding in an optimized buffered environment (Vallejo and Rinas 2004; Singh and Panda 2005). This has been discussed in detail in Chapter 1 under section 5.4. The baculovirus-insect expression system itself and maintenance *thereof* is much more expensive than the prokaryotic expression system, however the cost is often weighed against the demand and need for the product (Liljeqvist and Stahl 1999).

The baculovirus-insect expression system has been used in previous studies in an effort to produce a BTV subunit vaccine. These studies have been focused on the use of VP2 of BTV either by itself or in VLPs (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). The VLPs are an effective vaccine strategy since the viral particles lack nucleic acids and are presented in a “more” native conformation however it is difficult to express more than one protein simultaneously in the same expression system. These proteins have to be expressed at a more-or-less equal quantity. VLPs are also difficult to produce on a large scale with the particles not being necessarily stable and convenient for long-term storage (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). An alternative and also safe effective subunit vaccine is the use of the outer capsid protein VP2 by itself. In 1987, Huismans, et al. demonstrated BTV neutralization in sheep using isolated BTV-VP2 from intact viral particles while in 1990, Roy et al. demonstrated the vaccine potential of baculovirus-Sf9 expressed BTV(10)-VP2 in sheep (Huismans, van der Walt et

al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). The use BTV-VP2 by itself requires a protein expression system which produces large quantities of soluble BTV-VP2. Solubility is important as it is necessary for the expressed antigen to fold in the native conformation thereby exposing the viral neutralizing epitopes which are usually apart of the hydrophilic regions of the protein (Dinner, Sali et al. 2000; Dobson 2003). The problem with expression of large proteins such as BTV-VP2 is that they tend to interact non-natively and result in aggregation. Aggregated or insoluble antigens generally do not elicit a strong immune response. Insolubility can be dealt with by the expression of truncated versions of a particular antigen. These truncated antigens are designed such that hydrophobic regions which promote aggregation are deleted but they still contain the major neutralizing epitopes (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

The following chapter describes the baculovirus-Sf9 expression of full-length BTV-VP2 and truncated versions of the protein namely BTV(10)-VP2(aa450) and BTV(10)-VP2(aa650) designed in Chapter 2. The aim was to investigate the solubility of the expressed recombinant proteins and the effect of L-arginine on reversing aggregation. Solubility was assessed by high speed centrifugation and sucrose gradient ultracentrifugation. Once again all analysis will be carried out on BTV serotype 10, an available strain currently in circulation.

2. MATERIALS AND METHODS

2.1. Cloning into baculovirus expression vectors pDEST8™

The PCR amplified full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) (Chapter 2, section 2.2) were cloned into an entry vector pCR®8/GW/TOPO® (Invitrogen™) and then recombined into baculovirus-insect expression vector pDEST™ 8 by enzyme clonase II (Invitrogen™) according to the protocol described in Chapter 2, section 2.8. With the pDEST™ 8 expression vector (Figure 25) the recombinant BTV(10)-VP2 proteins can be expressed in insect cells without a fusion tag. The vector possesses a mini-Tn7 expression cassette containing a gentamicin resistance gene, an AcMNPV polyhedrin promoter and a Gateway® cloning cassette.

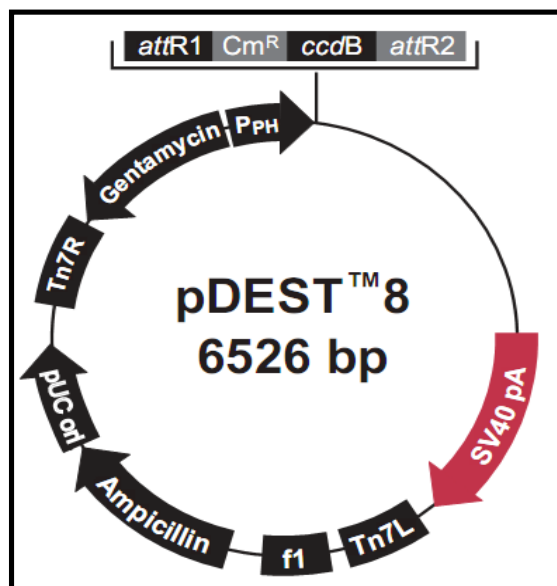


Figure 25. Map of baculovirus expression vector pDEST™ 8 (Bac-to-Bac® Expression manual from Invitrogen™).

2.2. Transposition of recombinant target DNA inserts from the expression vector into bacmid DNA of DH10Bac™

Transformation of recombinant pDEST8™ into DH10Bac™ was performed as per the specifications of the Bac-toBac® Baculovirus Expression System manual from Invitrogen™. The reaction was as follows:

Approximately 100 ng of recombinant pDEST8™ was added to 100 µl chemically competent DH10Bac™ and placed on ice for 30 minutes to promote DNA binding to the cell membrane. The cells were then heat-shocked without shaking at 42°C for 90 seconds to increase the permeability of the cells and promote the uptake of the recombinant vector. This was followed by 2 minutes incubation on ice to allow the cells to recover. Thereafter 900 µl of sterile LB broth was added to the cells which were then incubated at 37°C with shaking (220 rpm) for 4 hours. The incubation was necessary to allow for transposition of the target gene into the bacmid and for cell growth. After 4 hours, a 10-fold serial dilution of the cells were made using sterile LB broth and 100 µl of each dilution was spread on agar plates containing 100µg/ml X-Gal, 40µg/ml IPTG, 50µg/ml Gentamicin, 10µg/ml Kanamycin sulfate and 10µg/ml Tetracycline. The plates were incubated for 48 hours at 37°C. DH10Bac™ containing the recombinant bacmid was selected based on blue/white colony screening. Colonies displaying a white phenotype were selected and re-streaked on agar plates containing appropriate antibiotics, 100µg/ml X-Gal and 40µg/ml IPTG in order to verify the phenotype.

2.3. Isolation of recombinant bacmid DNA

The isolation of the recombinant bacmid DNA from DH10Bac™ was carried out as per the protocol outlined in the Invitrogen™ Bac-to-Bac® Baculovirus Expression System manual. The DH10Bac™ displaying a white phenotype (as verified above) was selected and grown in 5 ml LB broth containing 50µg/ml Gentamicine, 10µg/ml Kanamycin sulfate and 10µg/ml Tetracycline for 18 hours at 37°C with shaking (220 rpm). Cells from 1.5 ml of LB broth were collected by centrifugation at 6 000 x g in an Eppendorf 5415D desktop centrifuge for 10 minutes. The cells were then resuspended gently in 300 µl Lysis buffer (15 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A) and 300 µl 200mM NaOH/1% SDS and incubated at room temperature for 5 minutes. The genomic DNA and proteins were thereafter precipitated by the addition of 300 µl of 3 M Potassium Acetate (pH 5.5). The mixture was incubated on ice for a further 10 minutes and then centrifuged at 16000 x g for 10 minutes. The supernatant was transferred to a sterile Eppendorf to which 800 µl of Propan-2-ol was added. The Eppendorf was placed on ice for 10 minutes to allow for the precipitation of the bacmid. The bacmid was collected by centrifugation at 16 000 x g for 10 minutes. The pellet was then washed twice with 70% ethanol (500 µl) and air dried in a sterile Laminar flow. To avoid the shearing of the DNA, 40 µl UHQ was added to the pellet and the DNA was allowed to dissolve for 30 minutes on ice.

The transposition of the DNA insert into the bacmid was verified by PCR using full-length BTV(10)-VP2, BTV(10)-VP2(aa650) and BTV(10)-VP2(aa450) specific primers (Table 2) and PCR reaction and cycling conditions listed above (Chapter 2, section 2.2).

2.4. Transfection of Sf9 cells with recombinant bacmid DNA

The transfection of the Sf9 cells with the recombinant bacmid DNA was carried out in 35 mm wells of a 6 well plate. Each well was seeded with approximately 1.6×10^6 cells in TC-100 Insect Medium supplemented with 1.2% penicillin, streptomycin and fungizone as well as 10% (v/v) fetal calf serum (Highveld Biological, Sandton). The cells were then incubated at 27°C for 1-2 hours to allow for the attachment. Simultaneously, a bacmid-lipid complex was made up. Solution A, containing 6 µl of isolated recombinant bacmid (Section 2.12.2) was mixed with 100 µl of un-supplemented TC-100 Medium and Solution B, containing a mixture of 6 µl CELLFECTIN™ reagent (Invitrogen) and 100µl of un-supplemented TC-100 Medium were gently mixed and incubated at room temperature for 1 hour before the volume was made up to 1 ml with un-supplemented TC-100 Medium. The Sf9 cells were washed twice with 2 ml TC-100 medium and thereafter overlaid with the bacmid-lipid complex. The cells were incubated at 27°C for 12 hours so that the transfection could take place. The transfection mixture was then removed and the cells were overlaid with 2 ml of TC-100 Medium

supplemented with 1.2% penicillin/streptomycin/fungizone and 10% (v/v) fetal calf serum (Highveld Biological, Sandton). After 120 hours at 27°C, the recombinant baculovirus present in the supernatant was harvested by centrifugation at Beckman Coulter Avanti® J-E centrifuge using the JS-5.3 rotor. Approximately 200 µl of the supernatant containing the recombinant baculovirus was added to Sf9 cells overlaid with 1800 µl supplemented TC-100 Medium in a 6 well plate and incubated for a further 120 hours at 27°C. The Sf9 cells were collected by centrifugation (as per previous conditions) and analyzed with SDS-PAGE and immunoblot to confirm recombinant BTV(10)-VP2 protein expression while the baculovirus was plaque purified. Excess recombinant baculovirus in media was stored at 4°C and was protected from direct light.

2.5. Recombinant baculovirus plaque purification, virus titration and amplification

2.5.1. Baculovirus plaque purification

A six well plate was seeded with 1.6×10^6 Sf9 cells and incubated at 27°C for an hour to allow for cell attachment. The recombinant baculovirus in media was diluted in a ten-fold series with TC-100 Medium supplemented with 1.2% penicillin/streptomycin/fungizone and 10% (v/v) fetal calf serum (Highveld Biological, Sandton). The TC-100 medium was removed from the Sf9 cells and the cells overlaid with 500 µl of each dilution. The infection proceeded at room temperature for 1 hour before the virus containing medium was replaced with 2 ml of a 1% agarose (1% (w/v) Agarose Type VII (Sigma):Double strength Grace's medium (Highveld Biological, Sandton)). The cells were incubated for 10 days at 27°C followed by selection of single plaques which were re-suspended in with TC-100 Medium supplemented with 1.2% penicillin/streptomycin/fungizone and 10% (v/v) fetal calf serum (Highveld Biological, Sandton). Sf9 cells, seeded at 1.6×10^6 cells per 35 mm well, were infected with the purified recombinant baculovirus and incubated 27°C. After 72 hours, the cells were collected by centrifugation (6 000 x g for 10 minutes in an Eppendorf 5415D desktop centrifuge) and analyzed on SDS-PAGE for recombinant protein expression. The recombinant baculovirus in supernatant was titred and used for virus amplification.

2.5.2. Baculovirus titration

The recombinant baculovirus titration procedure was essentially the same as the plaque purification except with the amendment that after 10 days at 27°C, the cells overlaid in agarose was stained with 0.1% w/v Thiazolyl Blue Tetrazodium Bromide (Sigma) in PBS (Phosphate Buffered Saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) (500 µl/well) for approximately 1 hour. Baculovirus titre (pfu/ml) was then calculated as 2 x number of plaques x dilution factor.

2.5.3. Amplification of recombinant baculovirus stock

The recombinant baculovirus was amplified in 1×10^8 Sf9 cells in suspension in 100 ml TC-100 Medium supplemented with 1.2% penicillin/streptomycin/fungizone and 10% (v/v) fetal calf serum (Highveld Biological, Sandton). The Sf9 cells were infected at a multiplicity of infection (M.O.I) of 0.1 as per the Bac-to-Bac® Baculovirus Expression System manual (Invitrogen™) and maintained in Pyrex flasks kept at 27°C for 10 days with gentle shaking (approximately 100 rpm). The recombinant baculovirus in media was harvested by centrifugation at $6\,000 \times g$ for 10 minutes in a Beckman Coulter Avanti® J-E centrifuge using rotor JS-5.3. The virus stock was once again titred and stored at 4°C for short-term storage or -80°C for long-term storage.

2.6. Baculovirus-Sf9 expression of BTV-VP2 based proteins

Spodoptera frugiperda (Sf9) cells, purchased from the American Type Cell Collection, were used to express the baculovirus encoded proteins. The Sf9 cells were maintained as spinner cultures in Pyrex flasks at 27°C in TC100 Insect Medium supplemented with 10% (v/v) foetal calf serum, 1.2% penicillin, streptomycin and Fungizone and 0.8% pleuronic (Highveld Biological, Sandton).

All baculovirus protein expression for the purpose of this project was conducted on confluent monolayers of Sf9 cells (1.0×10^7) cultured in 75 cm² cell culture flasks. The cells were infected with the recombinant baculovirus at a M.O.I of 5 and were maintained at 27°C for 42 hours p.i.

To harvest the baculovirus infected cells, the culture flask were shaken vigorously to loosen the cells. The cells were then collected in 50 ml Falcon tubes by centrifugation at 4000 rpm for 5 minutes using a Beckman Coulter Avanti® J-E centrifuge using the JS-5.3 rotor. The medium was then discarded and the cell pellet was stored at -20°C until needed.

To extract the recombinant proteins from the Sf9 cells, the cell pellet (1.0×10^7 cells) was thawed out at room temperature and resuspended in 800 µl cold TNET buffer (50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA and 1% Triton X-100) containing 0.7 µg/ml Pepstatin (Roche) and 1mg/ml Pefabloc (Roche) and incubated at 4°C for 30 minutes. The cells were then homogenized with a glass Dounce homogenizer for a maximum of 20 times. The Sf9 cell lysate was then fractionated either by high speed centrifugation or with sucrose gradient ultracentrifugation and analyzed with SDS-PAGE and immunoblotting as described in Chapter 2, section 2.12.

For experimentation regarding arginine the Sf9 pellet (1.0×10^7 cells) was resuspended in 400 µl TNET buffer containing protease inhibitors and incubated at 4°C for 30 minutes. The cells were lysed by passing the mixture 10 times through a standard U-100 insulin needle. Arginine in Tris-HCl

(pH 8.0) was added to the cell lysate to a final concentration of 1.0 M and incubated at room temperature for 1 hour. The lysate was also separated either by high speed centrifugation or with sucrose gradient ultracentrifugation and analyzed with SDS-PAGE and immunoblotting as described in Chapter 2, section 2.12.

If the particulate fraction containing the insoluble recombinant proteins were to be analyzed with sucrose gradient ultracentrifugation, the following protocol was used:

The Sf9 cell lysate was layered on 2 ml 40% sucrose and centrifuged at 15 000 rpm for 20 minutes. The particulate was then resuspended in 800 µl PBS and the above protocol describing untreated or arginine treated lysates were followed.

3. RESULTS

3.1. Transposition of target DNA inserts from the baculovirus expression vector into baculovirus genome of DH10Bac™

For the recombinant baculovirus expression in Sf9 cells, the target genes, cloned into an appropriate baculovirus expression vector (pDEST™8) (Chapter 2, Section 3.3.2) has to be transposed into the bacmid DNA of DH10Bac™. The recombinant bacmids are then isolated and used to transfect Sf9 cells. The resulting recombinant baculovirus are then used for host cell infection for protein expression.

In Chapter 2, the PCR amplified BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) DNA inserts were cloned into the Gateway® entry vector pCR®8/GW/TOPO®. The presence of the DNA inserts and sequence homology to the BTV(10)-VP2 reference was confirmed by restriction endonuclease digestion and DNA sequencing respectively (Chapter 2, section 3.3.1 and Appendix A respectively). The target genes were then transferred to the baculovirus expression vector pDEST™ 8 by a recombination reaction between the attL and attR sites of pCR®8/GW/TOPO® and pDEST™ 8 vectors. The recombination reaction catalyzed by enzyme Gateway® Clonase™ II (Invitrogen™) allows for the maintenance of the orientation and reading frame of the target gene. The recombinant pDEST™ 8 was then transformed into DH10Bac™ cells. In the DH10Bac™ cells, a transposition reaction between the recombinant pDEST™ 8 mini-Tn7 site and the DH10Bac™ bacmid mini-attTn7 site result in the transfer of the entire expression cassette into the bacmid DNA. The recombinant bacmid DNA therefore contains the polyhedron promoter upstream of the target gene which allows for the expression of the target gene in Sf9 insect cells.

Recombinant DH10Bac™ cells were screened via blue-white colony selection and the putative recombinant bacmid DNA was isolated from a single white colony. The presence of the target genes within the bacmid DNA was confirmed by PCR using insert-specific primers (Table 2) and analyzed on 1% agarose gel stained with ethidium bromide (Figure 26).

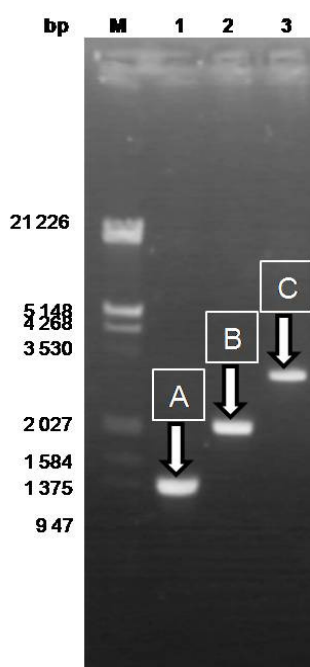


Figure 26. Confirmation of BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) gene inserts within isolated bacmid DNA with PCR using insert-specific primers. M, λ EcoRI/HindIII DNA Ladder (Fermentas™); Lane 1, 1365 bp BTV(10) agarose gel-purified VP2(aa450) amplicon (A); Lane 2, 1974 bp BTV(10) agarose gel-purified VP2(aa650) amplicon (B); Lane 3, 2883 bp BTV(10) agarose gel-purified VP2(ORF) amplicon (C).

Transposition of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) DNA insert into the bacmid genome was verified by PCR using insert-specific primers (Table 2). The PCR products were analyzed on a 1% agarose gel stained with ethidium bromide (Figure 26). DNA fragments with molecular weight of approximately 1365 (A), 1974 (B) and 2883 (C) bp were generated which corresponded to the theoretical molecular weight of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) respectively. Sf9 cells were thereafter transfected with the isolated bacmid DNA (which was used for the above PCR verification) in order to generate the recombinant baculovirus.

The amplified baculovirus containing inserted genes encoding BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) was used for all downstream baculovirus-Sf9 protein expression.

3.2. Pilot expression of recombinant BTV(10)-VP2 proteins using the baculovirus-Sf9 expression system

A pilot expression study was conducted to confirm expression of the BTV-VP2 based recombinant proteins. Sf9 cells (1.0×10^7) were infected with recombinant baculovirus at a MOI of 5. The cells were maintained at 27°C for 42 hours and lysed according to the protocol described under Section 2.6 of the current chapter. The soluble and insoluble proteins present in the lysate were separated by high speed centrifugation. Mock (Mk) uninfected Sf9 cell lysate and Sf9 cells infected with wild type (WT) baculovirus were used as controls. This was to ensure that the putative recombinant proteins were not Sf9 or baculovirus native proteins. Approximately 12 µl of each sample was analyzed with 12% SDS-PAGE stained with Coomassie G-250 (Figure 27).

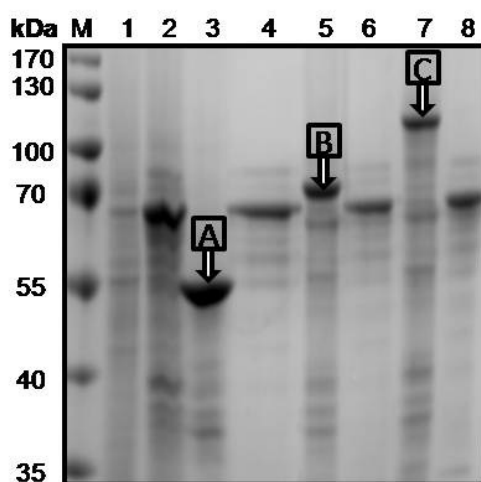


Figure 27. Recombinant baculovirus-Sf9 expression of recombinant BTV(10)-VP2 proteins harvested at 42 hours p.i. and analyzed on 12% SDS-polyacrylamide gel. M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, M_K uninfected Sf9 cell lysate; Lane 2, WT baculovirus infected Sf9 cell lysate; Lane 3 and 4, Sf9 cells expressing putative BTV(10)-VP2(aa450) pellet and supernatant respectively; Lane 5 and 6, Sf9 cells expressing putative BTV(10)-VP2(aa650) pellet and supernatant respectively; Lane 7 and 8, Sf9 cells expressing putative BTV(10)-VP2(ORF) pellet and supernatant respectively. 1.0×10^7 Sf9 cells expressing the recombinant proteins were used for the above analysis and approximately 12 µl of each sample was analyzed on the above polyacrylamide gel.

The analysis of the expressed putative recombinant BTV(10)-VP2 proteins on a 12% SDS-polyacrylamide gel revealed three proteins A, B and C corresponding to the theoretical molecular weights of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) respectively (Figure 27). These proteins were not present in the M_K uninfected Sf9 cell lysate nor were they present in the WT baculovirus infected Sf9 cell lysate.

To confirm the identity of the expression products, an immunoblot analysis of the pellet fractions was conducted (Figure 28). The proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membrane. Anti-BTV(10)-IgY antibodies were used as a primary detection antibody. Mk uninfected Sf9 cells and Sf9 cells infected with WT baculovirus were used as controls.

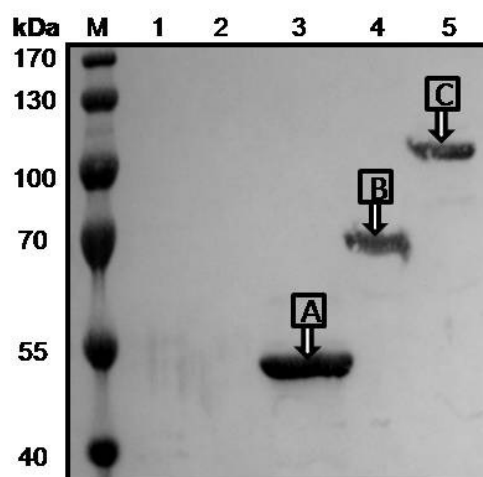


Figure 28. Immunoblot analysis of baculovirus-Sf9 expressed BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) using anti BTV(10)-IgY as a primary detection antibody. M, PageRuler™ Prestained Protein Ladder (Fermentas); Lane 1, Mk uninfected Sf9 cell lysate; Lane 2, WT baculovirus infected Sf9 cell lysate; Lane 3 Sf9 cells expressing BTV(10)-VP2(aa450) (pellet); Lane 4, Sf9 cells expressing BTV(10)-VP2(aa650) (pellet); Lane 5, Sf9 cells expressing BTV(10)-VP2(ORF) (pellet).

The immunoblot analysis of baculovirus-expressed BTV(10)-VP2 proteins using anti-BTV(10) IgY antibodies detected three proteins, A, B and C with molecular weights corresponding to the theoretical molecular weight of BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) respectively (Figure 28). These proteins were not detected in the Mk uninfected Sf9 cell lysate or the WT baculovirus infected Sf9 cell lysate.

By separating the soluble and insoluble proteins of the Sf9 cell lysate, Figure 27 also revealed that the recombinant BTV(10)-VP2 proteins were insoluble since they were only found in the insoluble fraction. Therefore the next step was to treat the Sf9 cell lysate with L-arginine to reverse aggregation of the BTV(10)-VP2 proteins.

3.3. Arginine treatment of Sf9 cell lysate containing the expressed recombinant BTV(10)-VP2 proteins

In the following experiment the Sf9 cell lysates containing the recombinant BTV(10)-VP2 proteins were treated with L-arginine to determine if it would reverse aggregation of the recombinant proteins.

Sf9 cells (1.0×10^7) were infected with recombinant baculovirus at a MOI of 5 and incubated at 27°C for 42 hours p.i. The cells were lysed and then treated with L-arginine to a final concentration of 1.0 M for 1 hour at room temperature. The proteins of the lysate were separated into the soluble and insoluble fractions using high speed centrifugation. Approximately 12 µl of each sample was analyzed with 12% SDS-PAGE (Figure 29).

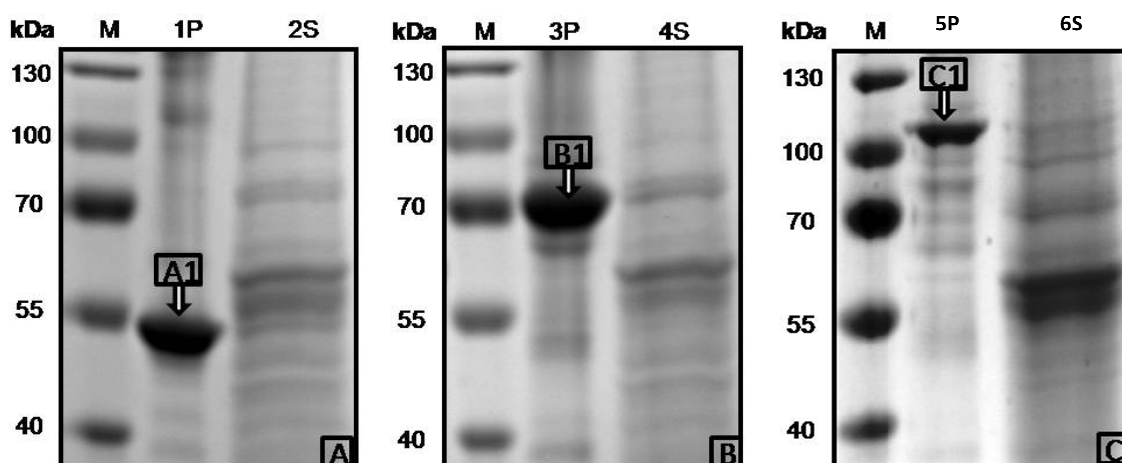


Figure 29. Treatment of Sf9 cell (1.0×10^7) lysate containing BTV(10)-VP2 (aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) harvested at 42 hours p.i. with 1 M L-arginine. M, PageRuler™ Prestained Protein Ladder (Fermentas); ; Lane 1P and 2S, arginine-treated Sf9 cells expressing BTV(10)-VP2(aa450) pellet and supernatant respectively; Lane 3P and 4S, arginine-treated Sf9 cells expressing BTV(10)-VP2(aa650) pellet and supernatant respectively; Lane 7P and 8S, arginine-treated Sf9 cells expressing BTV(10)-VP2(ORF) pellet and supernatant respectively. Approximately 12 µl of each sample was analyzed on the above polyacrylamide gel. **P and S denote pellet and supernatant respectively.**

According to Figure 29 total expressed BTV(10)-VP2 proteins remained insoluble (pellet) fraction after high speed centrifugation (A1, B1 and C1). Therefore L-arginine did not reverse aggregation of the recombinant BTV(10)-VP2 proteins.

In the next set of experiments, the recombinant BTV(10)-VP2 proteins were separated with sucrose gradient ultracentrifugation.

3.4. Sucrose gradient analysis of recombinant BTV(10)-VP2 proteins expressed in baculovirus infected Sf9 cells

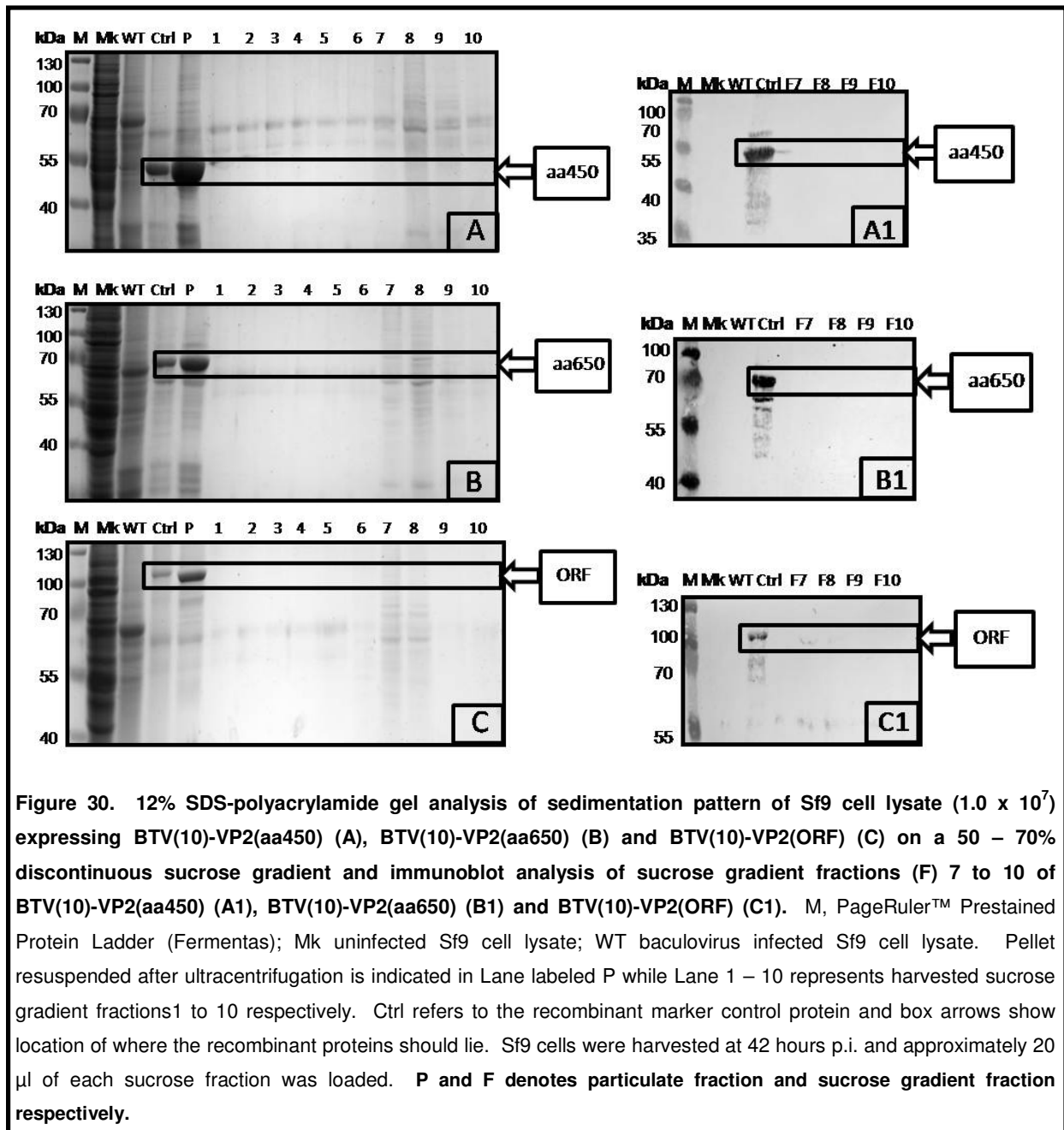
While high speed centrifugation may serve as a crude way to determine the solubility of a protein, the possibility of the putative soluble proteins remaining within the pellet fraction due to mass action still exists (Horn and Jackson 1972). A better suited technique for assaying solubility is sucrose gradient ultracentrifugation. With the sucrose gradient one is able to separate the soluble, partially aggregated and highly aggregated /insoluble proteins. Putative soluble proteins would be found on

the upper fractions of the gradient (fractions 7 to 10) while highly aggregated proteins, due to its high molecular weight would simply move through the gradient (fractions 1 to 6 and particulate fraction). The following set of experiments was based on sucrose gradient sedimentation analysis of untreated and arginine treated Sf9 cell lysate and particulate fractions.

3.4.1. Sucrose gradient sedimentation analysis of untreated Sf9 cell lysate containing the BTV(10)-VP2 proteins

In this particular experiment the aim was to determine the sedimentation pattern of **untreated** recombinant proteins from the Sf9 cell **lysate**. In this way it is possible to get a better indication of the solubility of the untreated recombinant BTV(10)-VP2 proteins present in the Sf9 cell lysate. Sf9 cells (1.0×10^7) were infected with the recombinant baculovirus at a MOI of 5. The cells were incubated at 27°C for 42 hours thereafter harvested and lysed. The resulting lysate was loaded onto a 50 – 70% sucrose gradient and centrifuged at 30 000 rpm for 18 hours at 4°C. Ten fractions of approximately 500 µl each were collected from the bottom to the top of the gradient and labeled as fractions 1 – 10 respectively. The particulate fraction was resuspended in STE and labeled P. Approximately 20 µl of each fraction was analyzed on 12% reducing SDS polyacrylamide gel stained with Coomassie blue G-250 (Figure 30, Panel A, B and C). Due to limited supply of anti-BTV(10)-IgY antibodies, only the sucrose gradient fractions 7 – 10 were analyzed with immunoblot for the presence of the recombinant proteins. F7 – F10 represents fractions 7 to 10 (Figure 30, Panel A1, B1 and C1).

No soluble recombinant proteins could be detected and all recombinant BTV(10)-VP2 proteins were found in the particulate fraction (Figure 30, Panel A, B and C, Lane P). Immunoblot analysis which identified the recombinant control proteins failed to detect any of the recombinant proteins in the sucrose gradient fractions 7 to 10 (Panel A1, B1 and C1).



The sedimentation pattern above is of the untreated Sf9 cell lysate containing the recombinant BTV(10)-VP2 proteins. Therefore the next aim was to determine whether L-arginine would reverse aggregation of the recombinant BTV(10)-VP2 proteins by treating the Sf9 cell lysate with L-arginine. The solubility of the recombinant proteins from the arginine treated lysate was analyzed with sucrose gradient ultracentrifugation.

3.4.2. Sucrose gradient sedimentation analysis of arginine treated Sf9 cell lysate containing the expressed BTV(10)-VP2 proteins

The aim of this particular experiment was to use sucrose gradient sedimentation analysis to investigate whether **L-arginine** would reverse the aggregation of the recombinant BTV(10)-VP2 proteins present in the Sf9 cell **lysate**.

Baculovirus-infected Sf9 cells (1.0×10^7) expressing the recombinant BTV(10)-VP2 proteins were lysed and treated with L-arginine to a final concentration of 1.0 M. The resulting treated lysate was loaded onto a 50 – 70% sucrose gradient and centrifuged at 30 000 rpm for 18 hour at 4°C. Approximately 20 µl of each of the 11 fractions collected thereafter including the pellet fraction was analyzed with SDS-PAGE and stained with Coomassie blue G-250 (Figure 31). Only sucrose gradient fractions 7 – 10 were analyzed with immunoblot for the presence of the recombinant proteins (Figure 31). Anti-BTV(10) IgY antibodies was used as a primary detection antibody. F7 – F10 represented fractions 7 to 10.

The arginine treatment solubilized a small fraction of the BTV(10)-VP2(aaa450) and BTV(10)-VP2(ORF) proteins which was only detectable by immunoblot analysis (Figure 31). These proteins were present in the sucrose gradient fractions 7 to 10 and 7 to 9 respectively (Panel A1 and C1 respectively). The bulk of these proteins still remained in the particulate fraction (Panel A and C, Lane P). No soluble BTV(10)-VP2(aaa650) proteins were detected after arginine treatment of Sf9 cell lysate. The recombinant protein was found in the particulate fraction after ultracentrifugation (Panel B, lane P). Further this protein was not detected with immunoblot analysis in sucrose gradient fractions 7 to 10 (Panel B1).

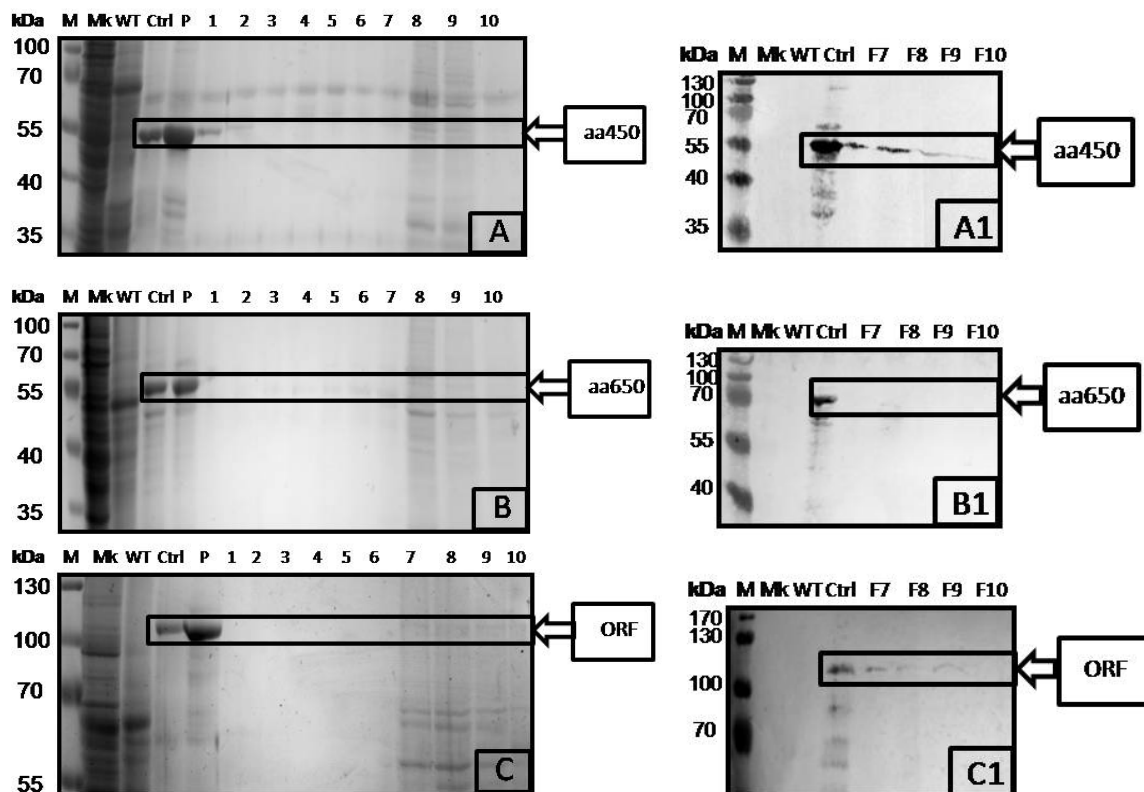


Figure 31. Sedimentation pattern of arginine treated (final concentration of 1.0 M) Sf9 cell (1.0×10^7) lysate containing BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) on a 50 – 70% discontinuous sucrose gradient and immunoblot analysis of sucrose gradient fractions (F) 7 to 10 of BTV(10)-VP2(aa450) (A1), BTV(10)-VP2(aa650) (B1) and BTV(10)-VP2(ORF) (C1). M, PageRuler™ Prestained Protein Ladder (Fermentas); Mk uninfected Sf9 cell lysate; WT baculovirus infected Sf9 cell lysate. Pellet resuspended after ultracentrifugation is indicated in Lane labeled P while Lane 1 – 10 represents harvested sucrose gradient fractions 1 to 10 respectively. Ctrl refers to the recombinant marker control protein and box arrows show location of where the recombinant proteins should lie. Sf9 cells were harvested at 42 hours p.i. and approximately 20 μ l of each sucrose fraction was loaded on a 12% reducing polyacrylamide gel. **P and F denotes particulate fraction and sucrose gradient fraction respectively.**

In the above experimentation, the sedimentation pattern of untreated and arginine treated Sf9 cell lysate was investigated. Previous experimentation has shown that a more reliable assessment of the sedimentation pattern of recombinant proteins is to look at the sedimentation pattern of the arginine treated particulate fraction (Henk Huismans, unpublished data, University of Pretoria). Therefore in the next set of experiments, the sedimentation pattern of untreated and arginine treated particulate fraction was investigated.

3.4.3. Sucrose gradient sedimentation analysis of particulate fraction containing BTV(10)-VP2 proteins

The aim here was to determine the sedimentation pattern of the **untreated particulate** fraction containing the expressed BTV(10)-VP2 proteins on a discontinuous sucrose gradient. This experiment serves as a control to the following experiment where L-arginine is used to try to solubilize the recombinant proteins of the particulate fraction.

The Sf9 cells (1.0×10^7) expressing the BTV(10)-VP2 proteins was lysed. The lysate was then loaded onto a 40% sucrose cushion and centrifuged at 15 000 rpm for 30 minutes. The host native soluble proteins in sucrose was discarded while the particulate was resuspended in PBS (800 μ l) and layered on a 50 – 70% sucrose gradient. Approximately 20 μ l of each of the 11 fractions (including particulate fraction) collected after ultracentrifugation at 30 000 x g for 18 hours was analyzed on 12% reducing SDS polyacrylamide gel stained with Coomassie blue G-250 (Figure 32, Panel A, B and C).

The recombinant proteins remained in the particulate fraction thus allowing this to serve as a suitable control for the following experiment (Figure 32, Panel A, B and C, Lane P). The next experimentation involved the treatment of the particulate fraction containing the recombinant proteins with L-arginine to determine if this treatment would reverse aggregation.

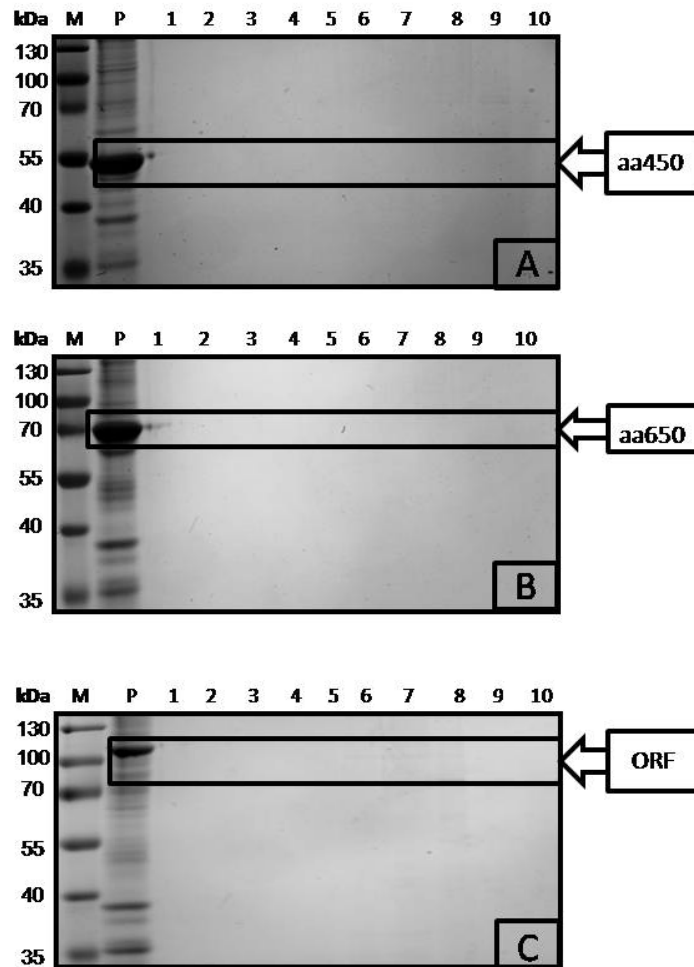


Figure 32. Analysis of sedimentation pattern of Sf9 pellet (1.0 x 10⁷) containing expressed BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) on a 50 – 70% discontinuous sucrose gradient. M, PageRuler™ Prestained Protein Ladder (Fermentas); Pellet resuspended after ultracentrifugation is indicated in Lane labeled P while Lane 1 – 10 represents harvested sucrose gradient fractions 1 to 10 respectively. Box arrows show location of where the recombinant proteins should lie. Sf9 cells were harvested at 42 hours p.i. and approximately 20 µl of each sucrose fraction was loaded on a 12% reducing polyacrylamide gel. **P denotes particulate fraction.**

3.4.4. Sucrose gradient sedimentation analysis of arginine treated particulate fraction containing the recombinant BTV(10)-VP2 proteins

The particulate fraction containing the recombinant BTV(10)-VP2 proteins was separated from the soluble proteins and treated with L-arginine. It is possible for the L-arginine to be used by the soluble proteins and therefore it was decided in this case to only treat the insoluble proteins of the particulate fraction. The aim of this experiment was to use sucrose gradient sedimentation analysis

to determine whether **L-arginine** would reverse aggregation of the BTV(10)-VP2 proteins in the **particulate** fraction allowing for the recovery of putative soluble recombinant proteins.

Baculovirus-infected Sf9 cells (1.0×10^7) containing expressed recombinant BTV(10)-VP2 proteins were lysed, layered on a 40% sucrose cushion and centrifuged at 15 000 rpm for 30 minutes. The pellet was resuspended in PBS (400 μ l) thereafter treated with L-arginine to a final concentration of 1.0 M and loaded onto a 50 – 70% sucrose gradient. The gradient was centrifuged at 30 000 rpm for 18 hours at 4°C. Approximately 20 μ l of each of the 11 fractions (including pellet) was thereafter collected and analyzed with SDS-PAGE stained with Coomassie blue G-250 (Figure 33).

The arginine treatment did not solubilize the recombinant proteins since total recombinant BTV(10)-VP2 proteins remained in the insoluble particulate fraction (Figure 33, Panel A, B and C, Lane P). Interestingly, after treatment with L-arginine, the background proteins of the insoluble fraction in Figure 32 were found in the sucrose fractions 9 and 10 while the BTV-VP2 based proteins remained in the particulate fraction. Therefore L-arginine effectively disaggregated these background proteins to release soluble proteins found on the upper sucrose fractions but failed to disaggregate the recombinant BTV(10)-VP2 proteins.

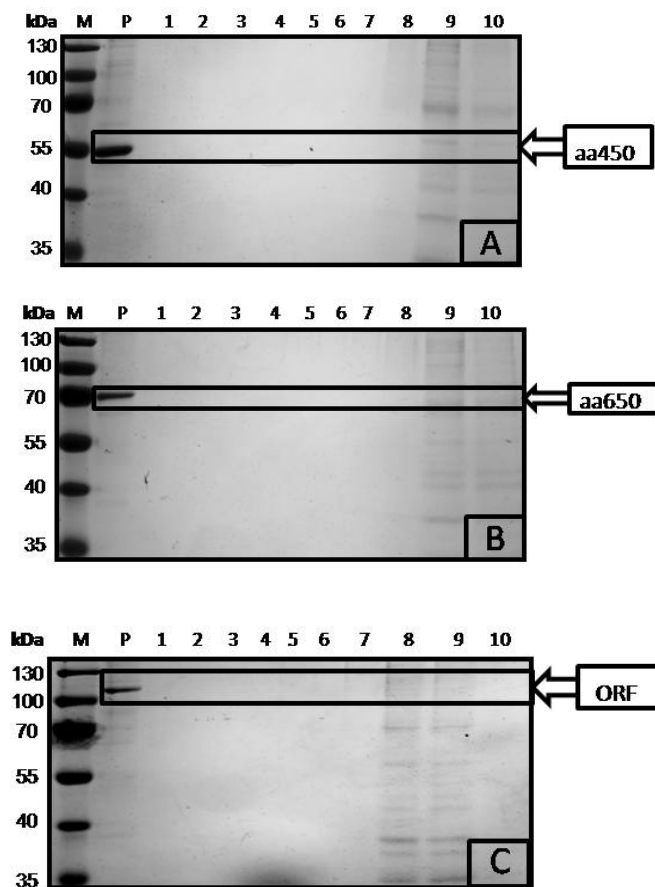


Figure 33. Sedimentation pattern of arginine treated (final concentration of 1.0 M) Sf9 cell (1.0×10^7) pellet containing BTV(10)-VP2(aa450) (A), BTV(10)-VP2(aa650) (B) and BTV(10)-VP2(ORF) (C) on a 50 – 70% discontinuous sucrose gradient. M, PageRuler™ Prestained Protein Ladder (Fermentas); Pellet resuspended after ultracentrifugation is indicated in Lane labeled P while Lane 1 – 10 represents harvested sucrose gradient fractions 1 to 10 respectively. Ctrl refers to the recombinant marker control protein and box arrows show location of where the recombinant proteins should lie. Sf9 cells were harvested at 42 hours p.i. and approximately 20 μ l of each sucrose fraction was loaded on a 12% reducing polyacrylamide gel. P denotes particulate fraction.

4. DISCUSSION

According to previous studies an effective BTV subunit vaccine strategy can either consist of the main neutralization-specific antigen VP2 by itself or as part of virus-like particles (VLPs) (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). The latter is advantageous due to a 'more' authentic viral structure being represented however the downside of this strategy is that high level production is difficult and expensive. The stability of VLPs also decreases over time (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). Numerous studies have already demonstrated the vaccine potential of BTV-VP2 by itself (Huismans,

van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). In this strategy solubility is of major importance. Solubility refers to the ability of the expressed protein to mimic the cognate antigen. This will in turn determine the effectiveness of the immune response since a soluble BTV-VP2 protein will expose hydrophilic regions which are likely to contain the virus neutralizing epitopes (Dinner, Sali et al. 2000; Dobson 2003). There is however difficulty associated with high level expression of soluble full-length proteins. This is mainly due to non-native interactions during *in vitro* synthesis which leads to aggregation. Aggregated proteins or insoluble proteins generally do not elicit a strong immune response. Aggregation may be overcome to an extent by expressing truncated versions of the antigen. These truncated antigens are designed so that they include the neutralizing epitopes but lack hydrophobic regions which might promote aggregation (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

The research presented in this chapter was specifically focused on the solubility analysis of BTV(10)-VP2 and truncated versions of this protein which was synthesized in a baculovirus-Sf9 expression system. In addition, this chapter also described the arginine treatment of the Sf9 cell lysate or pellet containing the recombinant proteins. Solubility was assessed by two separation techniques i.e. high speed centrifugation and sucrose gradient ultracentrifugation.

The truncated BTV(10)-VP2 proteins designed in Chapter 2 were expressed in the current chapter with the baculovirus-Sf9 expression system. These two truncated BTV(10)-VP2 proteins i.e. BTV(10)-VP2(aa450) (amino acid 1 to 450) and BTV(10)-VP2(aa650) (amino acid 1 to 650) contained BTV neutralizing epitopes and deleted C-terminal hydrophobic regions (White and Eaton 1990; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; DeMaula, Bonneau et al. 2000; Bhattacharya, Noad et al. 2007; Schwartz-Cornil, Mertens et al. 2008). While the smaller truncated protein contained predominantly the BTV neutralizing epitopes, the BTV(10)-VP2(aa650) protein contained in addition to the neutralizing epitopes, more of the BTV(10)-VP2 backbone. The hydrophobicity of the BTV(10)-VP2(aa650) protein was still essentially lower than the full-length BTV(10)-VP2.

Full-length BTV(10)-VP2 and the truncated versions were cloned into the baculovirus genome to generate recombinant viruses. The recombinant baculovirus was used to express the recombinant BTV(10)-VP2 proteins in Sf9 host cells. A pilot expression study was conducted to confirm expression of the recombinant BTV(10)-VP2 proteins. This was done with reducing SDS-PAGE and immunoblot analysis using anti-BTV(10)-IgY antibodies. The study revealed high level expression of untagged BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) corresponding to the theoretical molecular weight of 51, 66 and 110 kDa respectively (Figure 27 and Figure 28). The

recombinant proteins were synthesized at high levels from an initial 1.0×10^7 Sf9 cells infected with baculovirus at a low MOI of 5.

In addition to confirming expression of the recombinant BTV(10)-VP2 proteins, the pilot study also revealed that the recombinant proteins were insoluble since they were in the insoluble fraction (pellet) after high speed centrifugation (Figure 27). Several studies have shown that the high level expression with the baculovirus-insect expression system results in the accumulation of recombinant proteins as aggregates (Hsu and Betenbaugh 1997; Lenhard and Reilander 1997; Ailor and Betenbaugh 1999). Baculovirus infection of insect cells is usually followed by a shutdown of the host proteins synthesis. These host proteins include cellular proteins of the secretory pathway involved with folding, subunit assembly and post-translational modifications (Ailor and Betenbaugh 1999). The shortage of such proteins combined with the strong baculovirus polyhedron promoter leads to an accumulation of partially folded and/or misfolded polypeptides in the cytosol. This causes a 'crowding effect' thereby promoting intra molecular non-native and inter molecular associations by an increase in the association constants resulting in protein aggregation (van den Berg, Ellis et al. 1999).

Protein insolubility may also be the result of the presence non-native or the absence of disulfide bonds within the recombinant BTV-VP2 proteins. Various studies have demonstrated the formation of disulfide bridges within the host Sf9 cells (Duhe, Nielsen et al. 1994; Ou, Bergeron et al. 1995). Disulfide bridge formation takes place within the oxidizing environment of the rough endoplasmic reticulum (ER) due to the presence of enzymes such as protein disulfide isomerase (PDI) (Tu, Ho-Schleyer et al. 2000). It is highly probable that during high level expression non-native disulfide bonds form as a large number of polypeptide chains pass through the ER. Alternatively, due to the abundance of polypeptide chains, they are forced into the cytosol thereby preventing the proper disulfide bond formation in the reducing environment.

In the current study, no soluble recombinant BTV(10)-VP2 proteins were detected when the Sf9 cell lysate was treated with L-arginine followed by high speed centrifugation (Figure 29).

Despite the above mentioned disadvantages experienced during high level expression in baculovirus-Sf9 cells, the total insolubility associated with the BTV-VP2 based proteins even in the presence of L-arginine is somewhat questionable. Although the role of L-arginine in reversing and preventing aggregation is yet to be elucidated, it is hypothesized that this positively charged amino acid surrounds the proteins creating a repulsive force thereby reversing aggregation. The pI of arginine is 10.76 which is greater than the pH of the lysis buffer (pH 8.0) thus the amino acid should still maintain its positive charge and therefore be able to carry out its function. Recombinant proteins

will fold into a soluble conformation, however as time progresses the rapid increase in polypeptide chains leads to unfavorable interactions promoting aggregation (Henk Huismans, personal communication, University of Pretoria). Also if the recombinant BTV-VP2 proteins did possess disulfide bonds a small fraction of the expressed proteins would have folded correctly as it moved through the oxidizing environment of the ER.

Therefore it was necessary to critique the fractionation method which was being used thus far i.e. high speed centrifugation. Due to mass action, putative soluble proteins can be “pulled” with the insoluble fraction containing aggregated proteins during high speed centrifugation (Horn and Jackson 1972). This shortfall questions the validity of high speed centrifugation as a reliable method for investigating solubility. Therefore the solubility of the recombinant BTV(10)-VP2 proteins was also analyzed with sucrose gradient ultracentrifugation. The sedimentation pattern of the recombinant proteins provides a better indication of the aggregation status of the recombinant proteins by separating the soluble (found in sucrose fractions 7 to 10), partially aggregated and fully aggregated proteins (particulate and sucrose fractions 1 to 6).

The sedimentation pattern of untreated Sf9 cell lysate containing the BTV-VP2 based proteins showed that the total recombinant proteins were found in the particulate fraction (Figure 30). This correlated with the results obtained with high speed centrifugation where the total recombinant proteins were also present in the pellet.

However after treatment of the Sf9 cell lysate with L-arginine, the BTV(10)-VP2(aa450) and BTV(10)-VP2(ORF) proteins was present in the upper sucrose fractions (fractions 7 to 10 and 7 to 9 respectively) as well as the pellet and lower sucrose fractions (Figure 31). Therefore L-arginine was able to solubilize to some degree, a small fraction of the total BTV(10)-VP2(aa450) and BTV(10)-VP2(ORF) proteins by reversing aggregation of some of the recombinant proteins within the cell lysate. These soluble proteins were only detectable with immunoblot analysis of fractions 7 to 10 (Figure 31). The yield of these soluble proteins would not be practical for commercial vaccine purposes. By referring to mass action once again, it might explain the discrepancy between the results obtained with high speed centrifugation and sucrose gradient ultracentrifugation i.e. why small quantities of soluble proteins were not detected when the BTV(10)-VP2 proteins were treated with L-arginine and separated by high speed centrifugation (Figure 29) (data not shown for immunoblot analysis). The soluble proteins were probably “pulled” with the aggregated recombinant proteins to the insoluble fraction during high speed centrifugation (Horn and Jackson 1972). Although L-arginine solubilized a small amount of recombinant BTV(10)-VP2(aa450) and BTV(10)-VP2(ORF) proteins, the soluble amount however does not justify the cost associated with the

baculovirus-Sf9 expression system. Therefore in this case the expression system would be unsuitable for use in large scale vaccine production.

The arginine treatment of the Sf9 cell lysate rather than the Sf9 pellet containing the recombinant proteins was effective since in the latter all recombinant BTV(10)-VP2 proteins remained in the particulate fraction after sucrose gradient ultracentrifugation. An interesting observation was that after treatment with L-arginine, the insoluble proteins of the pellet fraction in Figure 32 were now found on at sucrose fractions 9 and 10. This suggests that arginine solubilized the previously insoluble background proteins in the upper sucrose fractions in Figure 33 while it failed to reverse aggregation of the recombinant BTV-VP2 proteins. This was probably due to the highly aggregated state of these proteins.

Although previous data showed that arginine treatment of the Sf9 cell lysate activated proteases causing degradation of the recombinant proteins or the arginine would be used by the host proteins (unpublished data, University of Pretoria), in the case of BTV(10)-VP2(aa450) and BTV(10)-VP2(ORF), the results suggest that treatment of the lysate is necessary to obtain a small fraction of soluble protein. Rather than a complete denaturation and refolding strategy of the insoluble proteins, we opted for the use of L-arginine since at this current time it was not possible to determine if the refolded proteins remained denatured or refolded successfully.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE PROSPECTS FOR THIS PROJECT

BTV has recently spread to previously disease free countries which prohibit the use of the current live-attenuated BTV vaccines due to safety concerns. This stresses the importance for new generation vaccines such as subunit vaccines (Ferrari, De Liberato et al. 2005; Veronesi, Hamblin et al. 2005). Subunit vaccines are an attractive vaccination strategy since it avoids the risks associated with live-attenuated vaccines (Russo, Turin et al. 1997; Liljeqvist and Stahl 1999). All BTV subunit vaccine strategies targets the outer capsid protein VP2 which is the main neutralization-specific antigen. As a subunit vaccine, BTV-VP2 may be used either by itself or as part of virus-like particles (VLPs) (Huismans, van der Walt et al. 1987; Roy, Urakawa et al. 1990; Roy, French et al. 1992; Roy, Bishop et al. 1994). VLPs have been shown to be an effective vaccine strategy but there are difficulties associated with large scale production and a reduced stability of the particles over time (Berg, Difatta et al. 2005; Wang, Zhao et al. 2006). When BTV-VP2 is used as a protein subunit vaccine then the antigen solubility is of a major importance. Solubility of an antigen refers to the ability of the protein to fold into an ordered tertiary conformation which authentically exposes the neutralizing epitopes to the immune system (Dinner, Sali et al. 2000; Dobson 2003). The immune response is generally poor against aggregated or insoluble proteins. To overcome insolubility one can truncate the antigen so that it contains predominantly major neutralizing epitopes but lack hydrophobic regions which promotes insolubility and aggregation during *in vitro* expression (Fukumoto, Xuan et al. 2003; Bonafe, Rininger et al. 2009; Liu, Zeng et al. 2009; Seo, Pyo et al. 2009).

Taking the above into account, this project specifically focused on the evaluation of the solubility of full-length BTV-VP2 and truncated versions of this protein expressed by either a prokaryotic and baculovirus-Sf9 expression system (Chapter 2 and 3 respectively). The reasons for choosing these particular expression systems are discussed in the relevant chapters. To achieve these aim, the full-length BTV(10)-VP2 protein and truncated versions of BTV(10)-VP2 i.e. BTV(10)-VP2(aa450) (amino acid 1 to 450) and BTV(10)-VP2(aa650) (amino acid 1 to 650) were expressed in a bacterial and baculovirus-Sf9 expression system. Hydrophobic regions at C-terminal of the BTV-VP2 protein which might contribute to aggregation during *in vitro* synthesis have been deleted. These truncated proteins however still contained BTV N-terminal neutralizing epitopes predicted from literature (White and Eaton 1990; DeMaula, Heidner et al. 1993; Pierce, Rossitto et al. 1995; DeMaula, Bonneau et al. 2000; Bhattacharya, Noad et al. 2007; Schwartz-Cornil, Mertens et al. 2008).

The full-length and truncated BTV(10)-VP2 proteins were expressed at high levels using both prokaryotic and baculovirus-Sf9 expression systems. These recombinant proteins were however only found in the insoluble fraction.

Arginine treatment of the Sf9 cell lysate to some degree reversed the aggregation of the BTV(10)-VP2(aa450) and full-length BTV(10)-VP2 proteins. However only a small fraction was solubilized which was only detectable with immunoblot analysis. One of the requirements for an ideal vaccine is that the vaccine should be produced in a cost-efficient method. Therefore the amount of soluble recombinant proteins produced by the baculovirus-Sf9 expression system was inadequate to justify the cost associated with this expression system.

An interesting find in this project was the synthesis of a soluble fraction of the recombinant BTV(10)-VP2 proteins, when these proteins were co-expressed with chaperones dnaK-dnaJ-GrpE in the prokaryotic expression system. This means that these chaperones were able to successfully fold some of the expressed BTV(10)-VP2 proteins. Approximately 50% of the total expressed BTV(10)-VP2(aa450) proteins were soluble while approximately 20% of the total expressed BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) proteins were soluble. Despite an unsuccessful purification of the soluble recombinant proteins by nickel affinity chromatography, the elution of these recombinant proteins through the column is verification of the proteins' solubility. Under ideal circumstances, a pure antigenic preparation is necessary for vaccine purposes. However various studies have demonstrated the vaccine potential of unpurified antigens expressed in the prokaryotic expression system. The outer-membrane lipopolysaccharide (LPS) component of the Gram negative bacteria serves as an effective adjuvant (Chedid, Audibert et al. 1976; Dimov, Ivanovska et al. 1992; Alving 1993; Trott, Hellestad et al. 2008). Additionally, there are various other biochemical protein purification methods such as size exclusion chromatography, immunoaffinity chromatography or sucrose gradient ultracentrifugation which may be successful at the isolation of these recombinant BTV(10)-VP2 proteins.

Nevertheless this is the first study to date demonstrating the synthesis of a soluble fraction of recombinant BTV(10)-VP2 proteins using the prokaryotic expression system. This expression system is ideal for vaccine purposes since it is an inexpensive method for rapid, large scale production of heterologous proteins. The yield of the soluble recombinant proteins in Figure 18 is from a 10 ml bacterial culture after 4 hours of expression. This yield of the soluble recombinant proteins is therefore not an optimized yield. In this regard, further investigation is required to determine the largest yield which may be obtained using this system. It is noteworthy that the arginine treatment of the bacterial lysate co-expressing dnaK-dnaJ-GrpE with the recombinant proteins did not increase the yield of the soluble fraction (data not shown). Alternatively one may

increase the soluble fraction of these recombinant proteins by co-expression of the dnaK-dnaJ-GrpE chaperones with the recombinant proteins in specialized bacterial strains such as Origami expression strains (Novagen). These strains contain an oxidizing cytosol which is conducive for efficient disulfide bond formation.

With the full-length BTV-VP2, the vaccine potential has already been demonstrated. Therefore in this regard it may just be a question of optimization to increase the soluble fraction of this recombinant protein. The important question is however whether or not the truncated BTV(10)-VP2 proteins are immunogenic and could serve as potential BTV subunit vaccines. Although it is possible to get a soluble fraction of the truncated BTV-VP2 proteins and these proteins do contain the BTV neutralizing epitopes, their tertiary folding is unknown. This implies that these proteins may not necessarily present the neutralizing epitopes in an authentic conformation to the immune system. However it should also be mentioned that in 2007, Athmaram et al. demonstrated the vaccine potential of a truncated BTV-VP2 protein in rabbits. This truncated protein was synthesized in the *Saccharomyces cerevisiae* (yeast) expression system (Athmaram, Bali et al. 2007). Therefore the next step is assessing the vaccine potential of these truncated BTV(10)-VP2 proteins.

Already mentioned above is that the yield of soluble recombinant BTV-VP2 proteins obtained with the baculovirus-Sf9 expression system was not cost-efficient for vaccine purposes. However due to the success obtained with the use of chaperones in the bacterial expression system, it might be interesting to determine the solubility of the recombinant BTV-VP2 proteins when co-expressed with chaperones in the baculovirus-Sf9 expression system.

Finally in this dissertation the solubility of the BTV-VP2 based proteins was assessed in the prokaryotic and baculovirus-Sf9 expression system. It may be necessary to assess the solubility of the recombinant BTV-VP2 proteins in a yeast and mammalian expression system. Once again the optimizations that gave positive results with the baculovirus-Sf9 and bacterial expression system may be applied to the yeast and mammalian expression system. In this way one may better compare which system is the most efficient for the synthesis of the soluble recombinant BTV(10)-VP2 proteins.

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APPENDIX A

Nucleotide sequence alignment of the target gene insert in pCR®8/GW/TOPO® with BTV(10)-VP2 reference sequence

Construct 1 – BTV(10)-VP2(aa450)

	10	20	30	40	50
BTV (10) –VP2 (aa450)	atggaggagtttcgtcataccagtgttctctgagagagatatcccatattc				
GW forward	ATGGAGGAGTTTCGTCATACCAGTGTTCCTCTGAGAGAGATATCCCATATTC				
GW reverse					
	60	70	80	90	100
BTV (10) –VP2 (aa450)	actgctgaaccactacccattggctatacggatagatgttaaagttagatg				
GW forward	ACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAGTTGATG				
GW reverse					
	110	120	130	140	150
BTV (10) –VP2 (aa450)	acgaagatggaagacataaccttattaagataacctgaatcagatatgatc				
GW forward	ACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGATATGATC				
GW reverse					
	160	170	180	190	200
BTV (10) –VP2 (aa450)	gacgtaccaaagttagtggtcatagaagctctaaattataggccaaaaag				
GW forward	GACGTACCAAAGTTGAGTGTTCATAGAAGCTCTAAATTATAGGCCAAAAAG				
GW reverse					
	210	220	230	240	250
BTV (10) –VP2 (aa450)	gaatgatggagtgggttggtgccgagattgctagatataacactacacgctt				
GW forward	GAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAACACTACACGCTT				
GW reverse					
	260	270	280	290	300
BTV (10) –VP2 (aa450)	atgacaagaggaaatccacgaaaagcgcgaaaggggttgaaatttacaaca				
GW forward	ATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGTTGAATTTACAACA				
GW reverse					
	310	320	330	340	350
BTV (10) –VP2 (aa450)	gatgctaagtggatgaaatgggcaatcgatgataagatggatatacaacc				
GW forward	GATGCTAAGTGGATGAAATGGGCAATCGATGATAAGATGGATATACAACC				
GW reverse					
	360	370	380	390	400
BTV (10) –VP2 (aa450)	tctcaagggttactttggataaatcactattccgtaaaccatcagctttttca				
GW forward	TCTCAAGGTTACTTTGGATAAATCACTATTCCGTAACCATCAGCTTTTCA				
GW reverse					
	410	420	430	440	450
					



BTV (10) –VP2 (aa450)
GW forward
GW reverse

attgtatagttaaaagcgaggtcagcaaatgCGGACACCATCTATTACGAC
ATTGTATAGTTAAAGCGAGGTCAGCAAAATGCGGACACCATCTATTACGAC

460 470 480 490 500

BTV (10) –VP2 (aa450)
GW forward
GW reverse

tattatccattggaaaatggagcaaagaaatgtaaccatacaaacctgga
TATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAACCTGGA

510 520 530 540 550

BTV (10) –VP2 (aa450)
GW forward
GW reverse

tttattacgaagtttaacaacgacagagatggtccacatcttacaggag
TTTATTACGAAGTTTAAACAACGACAGAGATGTTCCACATCTTACAGGGAG

560 570 580 590 600

BTV (10) –VP2 (aa450)
GW forward
GW reverse

cggtttatgccttaaaatcaacgcatagaactagtagctcattccgaaag
CGGCTTATGCCTTAAAATCAACGCATGAAGTAGTAGCTCATTCCGAAAGG

610 620 630 640 650

BTV (10) –VP2 (aa450)
GW forward
GW reverse

gaaagcacgagcgagacttatcagggttgggacgcagagatggatcacgct
GAAAGCACGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGATACAGCT
CAGAGATGGATACAGCT

660 670 680 690 700

BTV (10) –VP2 (aa450)
GW forward
GW reverse

gcgtaaagggtacaaagattgggtatagagggcagccgtacgaacgattca
GCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAACGATTCA
GCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAACGATTCA

710 720 730 740 750

BTV (10) –VP2 (aa450)
GW forward
GW reverse

tatccagcttaggttaagtaatacattaaaggaagagttccagatgagatt
TATCCAGCTTAGGTTCAAGTAATCATTAAAGGAAGAGTTCCAGATGAGATT
TATCCAGCTTAGGTTCAAGTAATCATTAAAGGAAGAGTTCCAGATGAGATT

760 770 780 790 800

BTV (10) –VP2 (aa450)
GW forward
GW reverse

agagacgaaatcgctgaacttaataaggattaaagatgaatggaaaaacgc
AGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATGGAAAAACGC
AGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATGGAAAAACGC

810 820 830 840 850

BTV (10) –VP2 (aa450)
GW forward
GW reverse

cgcgatgatagaactaaaatacgcgattggagctctgcaaaatactct
CGCGTATGATAGAACTAAAATACGCGCATTGGAGCTCTGCAAAATACTCT
CGCGTATGATAGAACTAAAATACGCGCATTGGAGCTCTGCAAAATACTCT

860 870 880 890 900

BTV (10) –VP2 (aa450)
GW forward
GW reverse

cgcgatcgagcgtaaaatggttgatggtcaagaggagccaaaggatgag
CGGCGATCGGACGTAAAATGTTGGATGTTCAAGAGGAGCCAAAGGATGAG
CGGCGATCGGACGTAAAATGTTGGATGTTCAAGAGGAGCCAAAGGATGAG



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          910      920      930      940      950
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             atggcactatccactagggtttcagttcaaattggacgagaaaattcattag
GW reverse             ATGGCACTATCCACTAGGTTTCAGTTCAAATTGGACGAGAAATTCATTAG

          960      970      980      990     1000
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             aaccgatcaagagcatgtaaacatatttgaagtaggtgggtcccgcgaacag
GW reverse             AACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCGCAACAG

          1010     1020     1030     1040     1050
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             atgatgggagggttttacgcgctgatcgctatagcggctaccgatacacaa
GW reverse             ATGATGGGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGATACACAA

          1060     1070     1080     1090     1100
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             caagggtcgcgtgtggaggacgaatccttatccgtgtctacgcggtgcgct
GW reverse             CAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACGCGGTGCGCT

          1110     1120     1130     1140     1150
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             aattgctgctgagtggtgagccttggtgacgtgtacttcacattgcgccaaa
GW reverse             AATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACATTGCGCCAAA

          1160     1170     1180     1190     1200
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             catataagtgagggtttgcgtccagaatatggtcaacgtgagaggccgcta
GW reverse             CATATAAGTGGAGTTTGCGTCCAGAATATGGTCAACGTGAGAGGCCGCTA

          1210     1220     1230     1240     1250
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             gaagataaataaatatgtattctctcgtcttaaatctgtttgacactaaactt
GW reverse             GAAGATAAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACACTAACTT

          1260     1270     1280     1290     1300
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             agctattggagatgaaatcattcactggcggatgaaaatttatcggccta
GW reverse             AGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATCGGCCTA

          1310     1320     1330     1340     1350
BTV (10) -VP2 (aa450)  ....|....|....|....|....|....|....|....|....|....|
GW forward             aagaaacaactcatgatgatggatatatttgtgtatcacagaaggatgat
GW reverse             AAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAGGATGAT

...

```



BTV(10)-VP2(aa450)

GW forward

GW reverse

TAG

Construct 2 – BTV(10)-VP2(aa650)

	10	20	30	40	50
BTV(10)-VP2(aa650)	CTCGAGATGGAGGAGTTTCGTCATACCAGTGTTCTCTGAGAGAGATATCCC				
GW forward 1	CTCGAGATGGAGGAGTTTCGTCATACCAGTGTTCTCTGAGAGAGATATCCC				
GW forward 2	CTCGAGATGGAGGAGTTTCGTCATACCAGTGTTCTCTGAGAGAGATATCCC				
Internal reverse 1					
Internal reverse 2					
GW reverse 1					
GW reverse 2					
	60	70	80	90	100
BTV(10)-VP2(aa650)	ATATTCACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAG				
GW forward 1	ATATTCACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAG				
GW forward 2	ATATTCACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAG				
Internal reverse 1					
Internal reverse 2					
GW reverse 1					
GW reverse 2					
	110	120	130	140	150
BTV(10)-VP2(aa650)	TTGATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT				
GW forward 1	TTGATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT				
GW forward 2	TTGATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT				
Internal reverse 1					
Internal reverse 2					
GW reverse 1					
GW reverse 2					
	160	170	180	190	200
BTV(10)-VP2(aa650)	ATGATCGACGTACCAAAGTTGAGTGT CATAGAAGCTCTAAATTATAGGCC				
GW forward 1	ATGATCGACGTACCAAAGTTGAGTGT CATAGAAGCTCTAAATTATAGGCC				
GW forward 2	ATGATCGACGTACCAAAGTTGAGTGT CATAGAAGCTCTAAATTATAGGCC				
Internal reverse 1					
Internal reverse 2					
GW reverse 1					
GW reverse 2					
	210	220	230	240	250
BTV(10)-VP2(aa650)	AAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAACACTAC				
GW forward 1	AAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAACACTAC				
GW forward 2	AAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAACACTAC				
Internal reverse 1					
Internal reverse 2					
GW reverse 1					
GW reverse 2					



```

                260       270       280       290       300
      ....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTGAAATTT
GW forward 1       ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTGAAATTT
GW forward 2       ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTGAAATTT
Internal reverse 1
Internal reverse 2
GW reverse 1
GW reverse 2

```

```

                310       320       330       340       350
      ....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) ACAACAGATGCTAAGTGGATGAAATGGGCAATCGATGATAAGATGGATAT
GW forward 1       ACAACAGATGCTAAGTGGATGAAATGGGCAATCGATGATAAGATGGATAT
GW forward 2       ACAACAGATGCTAAGTGGATGAAATGGGCAATCGATGATAAGATGGATAT
Internal reverse 1
Internal reverse 2
GW reverse 1
GW reverse 2

```

```

                360       370       380       390       400
      ....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCGGTAAACCATCAGC
GW forward 1       ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCGGTAAACCATCAGC
GW forward 2       ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCGGTAAACCATCAGC
Internal reverse 1
Internal reverse 2
GW reverse 1
GW reverse 2

```

```

                410       420       430       440       450
      ....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) TTTTCAATTGTATAGTTAAAGCGAGGTCAGCAAATGCGGACACCATCTAT
GW forward 1       TTTTCAATTGTATAGTTAAAGCGAGGTCAGCAAATGCGGACACCATCTAT
GW forward 2       TTTTCAATTGTATAGTTAAAGCGAGGTCAGCAAATGCGGACACCATCTAT
Internal reverse 1
Internal reverse 2
GW reverse 1
GW reverse 2

```

```

                460       470       480       490       500
      ....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
GW forward 1       TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
GW forward 2       TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
Internal reverse 1
Internal reverse 2
GW reverse 1
GW reverse 2

```

```

                510       520       530       540       550
      ....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) CCTGGATTTATTACGAAGTTTAAACAACGACAGAGATGTTCCACATCTTAC
GW forward 1       CCTGGATTTATTACGAAGTTTAAACAACGACAGAGATGTTCCACATCTTAC
GW forward 2       CCTGGATTTATTACGAAGTTTAAACAACGACAGAGATGTTCCACATCTTAC
Internal reverse 1

```



```
Internal reverse 2 CCTGGATTATTACGAAGTTTAAACAACGACAGAGATGTTCCACATCTTAC
GW reverse 1
GW reverse 2

          560      570      580      590      600
      ....|....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) AGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCATTCC
GW forward 1       AGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCATTCC
GW forward 2       AGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCATTCC
Internal reverse 1 AGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCATTCC
Internal reverse 2 AGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCATTCC
GW reverse 1
GW reverse 2

          610      620      630      640      650
      ....|....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) GAAAGGGAAAGCACGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
GW forward 1       GAAAGGGAAAGCACGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
GW forward 2       GAAAGGGAAAGCACGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
Internal reverse 1 GAAAGGGAAAGCACGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
Internal reverse 2 GAAAGGGAAAGCACGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
GW reverse 1
GW reverse 2

          660      670      680      690      700
      ....|....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
GW forward 1       ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
GW forward 2       ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
Internal reverse 1 ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
Internal reverse 2 ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
GW reverse 1
GW reverse 2

          710      720      730      740      750
      ....|....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) GATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
GW forward 1       GATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
GW forward 2       GATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
Internal reverse 1 GATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
Internal reverse 2 GATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
GW reverse 1
GW reverse 2

          760      770      780      790      800
      ....|....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) GAGATTAGAGACGAAATCGCTGAAC TTAATAGGATTAAAGATGAATGGAA
GW forward 1       GAGATTAGAGACGAAATCGCTGAAC TTAATAGGATTAAAGATGAATGGAA
GW forward 2       GAGATTAGAGACGAAATCGCTGAAC TTAATAGGATTAAAGATGAATGGAA
Internal reverse 1 GAGATTAGAGACGAAATCGCTGAAC TTAATAGGATTAAAGATGAATGGAA
Internal reverse 2 GAGATTAGAGACGAAATCGCTGAAC TTAATAGGATTAAAGATGAATGGAA
GW reverse 1
GW reverse 2

          810      820      830      840      850
      ....|....|....|....|....|....|....|....|....|....|
BTV(10)-VP2(aa650) AAACGCCGCGTATGATAGAACTAAAAATACGCCGATTGGAGCTCTGC AAAA
```



```
GW forward 1      AAACGCCGCGTATGATAGAACTAAAAACGCGCATTTGGAGCTCTGCAAAA
GW forward 2
Internal reverse 1 AAACGCCGCGTATGATAGAACTAAAAACGCGCATTTGGAGCTCTGCAAAA
Internal reverse 2 AAACGCCGCGTATGATAGAACTAAAAACGCGCATTTGGAGCTCTGCAAAA
GW reverse 1
GW reverse 2

      860      870      880      890      900
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
GW forward 1      TACTCTCGGCGATCGGACGTAAA
GW forward 2
Internal reverse 1 TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
Internal reverse 2 TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
GW reverse 1
GW reverse 2

      910      920      930      940      950
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTGGACGAGAAATT
GW forward 1
GW forward 2
Internal reverse 1 GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTGGACGAGAAATT
Internal reverse 2 GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTGGACGAGAAATT
GW reverse 1
GW reverse 2

      960      970      980      990      1000
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
GW forward 1
GW forward 2
Internal reverse 1 CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
Internal reverse 2 CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
GW reverse 1
GW reverse 2

      1010     1020     1030     1040     1050
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
GW forward 1
GW forward 2
Internal reverse 1 CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
Internal reverse 2 CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
GW reverse 1      TAGCGGCTACCGAT
GW reverse 2

      1060     1070     1080     1090     1100
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) ACACAACAAGGTCGCGTGTTGGAGGACGAATCCTTATCCGTGTCTACGCGG
GW forward 1
GW forward 2
Internal reverse 1 ACACAACAAGGTCGCGTGTTGGAGGACGAATCCTTATCCGTGTCTACGCGG
Internal reverse 2 ACACAACAAGGTCGCGTGTTGGAGGACGAATCCTTATCCGTGTCTACGCGG
GW reverse 1
GW reverse 2
```



```

      1110      1120      1130      1140      1150
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACATTGC
GW forward 1
GW forward 2
Internal reverse 1 TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACATTGC
Internal reverse 2 TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACATTGC
GW reverse 1
GW reverse 2

      1160      1170      1180      1190      1200
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) GCCAAACATATAAGTGGAGTTTGCCTCCAGAAATATGGTCAACGTGAGAGG
GW forward 1
GW forward 2
Internal reverse 1 GCCAAACATATAAGTGGAGTTTGCCTCCAGAAATATGGTCAACGTGAGAGG
Internal reverse 2 GCCAAACATATAAGTGGAGTTTGCCTCCAGAAATATGGTCAACGTGAGAGG
GW reverse 1
GW reverse 2

      1210      1220      1230      1240      1250
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) CCGCTAGAAGATAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACAC
GW forward 1
GW forward 2
Internal reverse 1 CCGCTAGAAGATAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACAC
Internal reverse 2 CCGCTAGAAGATAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACAC
GW reverse 1
GW reverse 2

      1260      1270      1280      1290      1300
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) TAACTTAGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATC
GW forward 1
GW forward 2
Internal reverse 1 TAACTTAGCTA
Internal reverse 2 TAACTTAGCTA
GW reverse 1 TAACTTAGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATC
GW reverse 2 TAACTTAGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATC

      1310      1320      1330      1340      1350
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) GGCCATAAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAG
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
GW reverse 1 GGCCATAAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAG
GW reverse 2 GGCCATAAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAG

      1360      1370      1380      1390      1400
      |...|...|...|...|...|...|...|...|...|...|
BTV(10)-VP2(aa650) GATGATGACGAATTATTATGTGAAATTGACGGGGATAGGTATAAAGAGAT
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
```



GW reverse 1 GATGATGACGAATTATTATGTGAAATTGACGGGGATAGGTATAAAGAGAT
GW reverse 2 GATGATGACGAATTATTATGTGAAATTGACGGGGATAGGTATAAAGAGAT

1410 1420 1430 1440 1450
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) GTTTGATCGTATGATTCAAGGCGGATGGGATCAGGAGCGATTTAAACTGC
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
GW reverse 1 GTTTGATCGTATGATTCAAGGCGGATGGGATCAGGAGCGATTTAAACTGC
GW reverse 2 GTTTGATCGTATGATTCAAGGCGGATGGGATCAGGAGCGATTTAAACTGC

1460 1470 1480 1490 1500
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) ACAGTATATTAACTGAACCAAACCTGCTTACTATCGACTTCGAGAAGGAT
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
GW reverse 1 ACAGTATATTAACTGAACCAAACCTGCTTACTATCGACTTCGAGAAGGAT
GW reverse 2 ACAGTATATTAACTGAACCAAACCTGCTTACTATCGACTTCGAGAAGGAT

1510 1520 1530 1540 1550
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) GCCTATCTTGGTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGACAA
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
GW reverse 1 GCCTATCTTGGTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGACAA
GW reverse 2 GCCTATCTTGGTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGACAA

1560 1570 1580 1590 1600
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) ATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTGGCG
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
GW reverse 1 ATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTGGCG
GW reverse 2 ATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTGGCG

1610 1620 1630 1640 1650
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) AGATCGCAACCCGGAAGGTGGATGACCCCTTGGAACAATCGAGCGGTTCAT
GW forward 1
GW forward 2
Internal reverse 1
Internal reverse 2
GW reverse 1 AGATCGCAACCCGGAAGGTGGATGACCCCTTGGAACAATCGAGCGGTTCAT
GW reverse 2 AGATCGCAACCCGGAAGGTGGATGACCCCTTGGAACAATCGAGCGGTTCAT

1660 1670 1680 1690 1700
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) GGGTACATTAAACGTCAGCGGAGTCTCTAGGGTATGTGTAGGGCCATA
GW forward 1



```
GW forward 2 -----
Internal reverse 1 -----
Internal reverse 2 -----
GW reverse 1 GGGTACATTAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCCATA
GW reverse 2 GGGTACATTAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCCATA

          1710      1720      1730      1740      1750
          .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) TTATGACCTACGTCCTGCAATTATTCTGGCGACGCTCTCAGTCTGGAAACAAA
GW forward 1 -----
GW forward 2 -----
Internal reverse 1 -----
Internal reverse 2 -----
GW reverse 1 TTATGACCTACGTCCTGCAATTATTCTGGCGACGCTCTCAGTCTGGAAACAAA
GW reverse 2 TTATGACCTACGTCCTGCAATTATTCTGGCGACGCTCTCAGTCTGGAAACAAA

          1760      1770      1780      1790      1800
          .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) AACAGTCAGCCGTATTCTGAATATATGGCTCAACAAGATGATTTCCCGCG
GW forward 1 -----
GW forward 2 -----
Internal reverse 1 -----
Internal reverse 2 -----
GW reverse 1 AACAGTCAGCCGTATTCTGAATATATGGCTCAACAAGATGATTTCCCGCG
GW reverse 2 AACAGTCAGCCGTATTCTGAATATATGGCTCAACAAGATGATTTCCCGCG

          1810      1820      1830      1840      1850
          .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) CTAACAGATTATACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGGAAC
GW forward 1 -----
GW forward 2 -----
Internal reverse 1 -----
Internal reverse 2 -----
GW reverse 1 CTAACAGATTATACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGGAAC
GW reverse 2 CTAACAGATTATACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGGAAC

          1860      1870      1880      1890      1900
          .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) CTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATGAAC
GW forward 1 -----
GW forward 2 -----
Internal reverse 1 -----
Internal reverse 2 -----
GW reverse 1 CTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATGAAC
GW reverse 2 CTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATGAAC

          1910      1920      1930      1940      1950
          .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(aa650) GATTGGACCCAAGTTTACATGAGGGGAGGGAGCATGAGACATACATGCAC
GW forward 1 -----
GW forward 2 -----
Internal reverse 1 -----
Internal reverse 2 -----
GW reverse 1 GATTGGACCCAAGTTTACATGAGGGGAGGGAGCATGAGACATACATGCAC
GW reverse 2 GATTGGACCCAAGTTTACATGAGGGGAGGGAGCATGAGACATACATGCAC

          1960      1970
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      .....|.....|.....|.....|.....
BTV(10)-VP2(aa650)  CCGGCAATCAATGATTAGTCTAGA
GW forward 1
GW forward 2
Internal reverse 1  -----
Internal reverse 2  -----
GW reverse 1       CCGGCAATCAATGATTAGTCTAGA
GW reverse 2       CCGGCAATCAATGATTAGTCTAGA

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Construct 3 – BTV(10)-VP2(ORF)

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      10      20      30      40      50
      .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(ORF)  CTCCGAGATGGAGGAGTTCGTCATACCAGTGTTCTCTGAGAGAGATATCCC
GW forward 1      CTCCGAGATGGAGGAGTTCGTCATACCAGTGTTCTCTGAGAGAGATATCCC
GW forward 2      CTCCGAGATGGAGGAGTTCGTCATACCAGTGTTCTCTGAGAGAGATATCCC
VP2 forward 1
VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

      60      70      80      90     100
      .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(ORF)  ATATTCACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAG
GW forward 1      ATATTCACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAG
GW forward 2      ATATTCACTGCTGAACCACTACCCATTGGCTATACGGATAGATGTTAAAG
VP2 forward 1
VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

      110     120     130     140     150
      .....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV(10)-VP2(ORF)  TTGATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT
GW forward 1      TTGATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT
GW forward 2      TTGATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT
VP2 forward 1      ATGACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT
VP2 forward 2      GACGAAGATGGAAGACATAACCTTATTAAGATACCTGAATCAGAT
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1

```

GW reverse 2

	160	170	180	190	200
BTV (10) -VP2 (ORF)	ATGATCGACGTACCAAAGTTGAGTGT	CATAGAAGCTCTAAATTATAGGCC			
GW forward 1	ATGATCGACGTACCAAAGTTGAGTGT	CATAGAAGCTCTAAATTATAGGCC			
GW forward 2	ATGATCGACGTACCAAAGTTGAGTGT	CATAGAAGCTCTAAATTATAGGCC			
VP2 forward 1	ATGATCGACGTACCAAAGTTGAGTGT	CATAGAAGCTCTAAATTATAGGCC			
VP2 forward 2	ATGATCGACGTACCAAAGTTGAGTGT	CATAGAAGCTCTAAATTATAGGCC			
VP2 internal 1	-----				
VP2 internal 2	-----				
VP2 internal 3	-----				
VP2 internal 4	-----				
VP2 reverse 1	-----				
VP2 reverse 2	-----				
GW reverse 1	-----				
GW reverse 2	-----				

	210	220	230	240	250
BTV(10)-VP2 (ORF)		AAAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAA	CACTAC		
GW forward 1	AAAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAA	CACTAC			
GW forward 2	AAAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAA	CACTAC			
VP2 forward 1	AAAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAA	CACTAC			
VP2 forward 2	AAAAAAGGAATGATGGAGTGGTTGTGCCGAGATTGCTAGATATAA	CACTAC			
VP2 internal 1	-----				
VP2 internal 2	-----				
VP2 internal 3	-----				
VP2 internal 4	-----				
VP2 reverse 1	-----				
VP2 reverse 2	-----				
GW reverse 1	-----				
GW reverse 2	-----				

	260	270	280	290	300
BTV(10)-VP2 (ORF)	ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTTGAATTT				
GW forward 1	ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTTGAATTT				
GW forward 2	ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTTGAATTT				
VP2 forward 1	ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTTGAATTT				
VP2 forward 2	ACGCTTATGACAAGAGGAAATCCACGAAAAGCGCGAAAGGGGTTGAATTT				
VP2 internal 1	-----GGGGTTGAATTT				
VP2 internal 2	-----				
VP2 internal 3	-----				
VP2 internal 4	-----				
VP2 reverse 1	-----				
VP2 reverse 2	-----				
GW reverse 1	-----				
GW reverse 2	-----				

310 320 330 340 350

BTV(10)-VP2 (ORF) ACAAACAGATGCTAAGTGGATGAAATGGGCCAATCGATGATAAGATGGATAT

GW forward 1 ACAAACAGATGCTAAGTGGATGAAATGGGCCAATCGATGATAAGATGGATAT

GW forward 2 ACAAACAGATGCTAAGTGGATGAAATGGGCCAATCGATGATAAGATGGATAT

VP2 forward 1 ACAAACAGATGCTAAGTGGATGAAATGGGCCAATCGATGATAAGATGGATAT

VP2 forward 2 ACAAACAGATGCTAAGTGGATGAAATGGGCCAATCGATGATAAGATGGATAT



VP2 internal 1	ACAACAGATGCTAAGTGGATGAAATGGGCAATCGATGATAAGATGGATAT
VP2 intrenal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	
	360 370 380 390 400
BTV(10)-VP2(ORF)	ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCCGTAAACCATCAGC
GW forward 1	ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCCGTAAACCATCAGC
GW forward 2	ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCCGTAAACCATCAGC
VP2 forward 1	ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCCGTAAACCATCAGC
VP2 forward 2	ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCCGTAAACCATCAGC
VP2 internal 1	ACAACCTCTCAAGGTTACTTTGGATAATCACTATTCCGTAAACCATCAGC
VP2 intrenal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	
	410 420 430 440 450
BTV(10)-VP2(ORF)	TTTTCAATTGTATAGTTAAAGCGAGGTGAGCAAAATGCGGACACCATCTAT
GW forward 1	TTTTCAATTGTATAGTTAAAGCGAGGTGAGCAAAATGCGGACACCATCTAT
GW forward 2	TTTTCAATTGTATAGTTAAAGCGAGGTGAGCAAAATGCGGACACCATCTAT
VP2 forward 1	TTTTCAATTGTATAGTTAAAGCGAGGTGAGCAAAATGCGGACACCATCTAT
VP2 forward 2	TTTTCAATTGTATAGTTAAAGCGAGGTGAGCAAAATGCGGACACCATCTAT
VP2 internal 1	TTTTCAATTGTATAGTTAAAGCGAGGTGAGCAAAATGCGGACACCATCTAT
VP2 intrenal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	
	460 470 480 490 500
BTV(10)-VP2(ORF)	TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
GW forward 1	TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
GW forward 2	TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
VP2 forward 1	TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
VP2 forward 2	TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
VP2 internal 1	TACGACTATTATCCATTGGAAAATGGAGCAAAGAAATGTAACCATACAAA
VP2 intrenal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	



	510520530540550
BTV (10) –VP2 (ORF)	CCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCTTAC
GW forward 1	CCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCTTAC
GW forward 2	CCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCTTAC
VP2 forward 1	CCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCTTAC
VP2 forward 2	CCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCTTAC
VP2 internal 1	CCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCTTAC
VP2 internal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	
	560570580590600
BTV (10) –VP2 (ORF)	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
GW forward 1	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
GW forward 2	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
VP2 forward 1	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
VP2 forward 2	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
VP2 internal 1	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
VP2 internal 2	AGGGAGCGGCTTATGCCTTAAATCAACGCATGAAC TAGTAGCTCATTCC
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	
	610620630640650
BTV (10) –VP2 (ORF)	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
GW forward 1	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
GW forward 2	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
VP2 forward 1	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
VP2 forward 2	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
VP2 internal 1	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
VP2 internal 2	GAAAGGGAAAGCAGAGCGAGACTTATCAGGTTGGGACGCAGAGATGGAT
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	
VP2 reverse 2	
GW reverse 1	
GW reverse 2	
	660670680690700
BTV (10) –VP2 (ORF)	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
GW forward 1	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
GW forward 2	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
VP2 forward 1	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
VP2 forward 2	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
VP2 internal 1	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC
VP2 internal 2	ACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACGAAC



VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	710 720 730 740 750
	
BTV (10) -VP2 (ORF)	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
GW forward 1	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
GW forward 2	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
VP2 forward 1	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
VP2 forward 2	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
VP2 internal 1	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
VP2 intrenal 2	GATTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCAGAT
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	760 770 780 790 800
	
BTV (10) -VP2 (ORF)	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
GW forward 1	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
GW forward 2	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
VP2 forward 1	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
VP2 forward 2	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
VP2 internal 1	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
VP2 intrenal 2	GAGATTAGAGACGAAATCGCTGAACTTAATAGGATTAAAGATGAATGGAA
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	810 820 830 840 850
	
BTV (10) -VP2 (ORF)	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
GW forward 1	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
GW forward 2	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
VP2 forward 1	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
VP2 forward 2	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
VP2 internal 1	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
VP2 intrenal 2	AAACGCCGCGTATGATAGAACTAAAAATACGCGCATTGGAGCTCTGCAAAA
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	860 870 880 890 900
	



BTV (10) -VP2 (ORF) TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
GW forward 1 TACTCTCGGCGATCGGACGTAAA
GW forward 2 TACTCTCGGCGATCGGACGTAAA
VP2 forward 1 TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
VP2 forward 2 TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
VP2 internal 1 TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
VP2 internal 2 TACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCAAAG
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

910 920 930 940 950
BTV (10) -VP2 (ORF) GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAAATT
GW forward 1
GW forward 2
VP2 forward 1 GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAAATT
VP2 forward 2 GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAAATT
VP2 internal 1 GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAAATT
VP2 internal 2 GATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAAATT
VP2 internal 3 CGAGAAATT
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

960 970 980 990 1000
BTV (10) -VP2 (ORF) CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
GW forward 1
GW forward 2
VP2 forward 1 CATTAGAACCGATCAAGAGCATGTAAACATATTT
VP2 forward 2 CATTAGAACCGATCAAGAGCATGTAAACATATTT
VP2 internal 1 CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
VP2 internal 2 CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
VP2 internal 3 CATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTCCCG
VP2 internal 4 AGGTGGTCCCG
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

1010 1020 1030 1040 1050
BTV (10) -VP2 (ORF) CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1 CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
VP2 internal 2 CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
VP2 internal 3 CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT
VP2 internal 4 CAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACCGAT



VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

```

                                     1060      1070      1080      1090      1100
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) -VP2 (ORF) ACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACGCGG
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1 ACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACGCGG
VP2 internal 2 ACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACGCGG
VP2 internal 3 ACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACGCGG
VP2 internal 4 ACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACGCGG
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2
```

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                                     1110      1120      1130      1140      1150
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) -VP2 (ORF) TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTTCACATTGC
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1 TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTTCACATTGC
VP2 internal 2 TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTTCACATTGC
VP2 internal 3 TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTTCACATTGC
VP2 internal 4 TGGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTTCACATTGC
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2
```

```

                                     1160      1170      1180      1190      1200
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) -VP2 (ORF) GCCAAACATATAAGTGGAGTTTGGCTCCAGAATATGGTCAACGTGAGAGG
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1 GCCAAACATATAAGTGGAGTTTGGCTCCAGAATATGGTCAACGTGAGAGG
VP2 internal 2 GCCAAACATATAAGTGGAGTTTGGCTCCAGAATATGGTCAACGTGAGAGG
VP2 internal 3 GCCAAACATATAAGTGGAGTTTGGCTCCAGAATATGGTCAACGTGAGAGG
VP2 internal 4 GCCAAACATATAAGTGGAGTTTGGCTCCAGAATATGGTCAACGTGAGAGG
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2
```

```

                                     1210      1220      1230      1240      1250
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) -VP2 (ORF) CCGCTAGAAGATAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACAC
GW forward 1
```



GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	CCGCTAGAAGATAATAAATA
VP2 internal 2	CCGCTAGAAGATAATAAATA
VP2 internal 3	CCGCTAGAAGATAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACAC
VP2 internal 4	CCGCTAGAAGATAATAAATATGTATTCTCTCGTCTTAATCTGTTTGACAC
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	1260 1270 1280 1290 1300
BTV (10) -VP2 (ORF)	
	TAACCTAGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATC
GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	TAACCTAGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATC
VP2 internal 4	TAACCTAGCTATTGGAGATGAAATCATTCACTGGCGGTATGAAATTTATC
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	1310 1320 1330 1340 1350
BTV (10) -VP2 (ORF)	
	GGCCTAAAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAG
GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	GGCCTAAAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAG
VP2 internal 4	GGCCTAAAGAAACAACCTCATGATGATGGATATATTTGTGTATCACAGAAG
VP2 reverse 1	-----
VP2 reverse 2	-----
GW reverse 1	-----
GW reverse 2	-----
	1360 1370 1380 1390 1400
BTV (10) -VP2 (ORF)	
	GATGATGACGAATTATTATGTGAAATTGACGGGGATAGGTATAAAGAGAT
GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	GATGATGACGAATTATTATGTGAAATTGACGGGGATAGGTATAAAGAGAT
VP2 internal 4	GATGATGACGAATTATTATGTGAAATTGACGGGGATAGGTATAAAGAGAT
VP2 reverse 1	-----
VP2 reverse 2	-----

```
GW reverse 1
GW reverse 2
```

1410 1420 1430 1440 1450

BTV (10) -VP2 (ORF) GTTTGATCGTATGATTCAAGGCCGATGGGATCAGGAGCGATTTAAACTGC

GW forward 1

GW forward 2

VP2 forward 1

VP2 forward 2

VP2 internal 1

VP2 internal 2

VP2 internal 3 GTTTGATCGTATGATTCAAGGCCGATGGGATCAGGAGCGATTTAAACTGC

VP2 internal 4 GTTTGATCGTATGATTCAAGGCCGATGGGATCAGGAGCGATTTAAACTGC

VP2 reverse 1

VP2 reverse 2

GW reverse 1

GW reverse 2

1460 1470 1480 1490 1500

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV (10) -VP2 (ORF) ACAGTATATTAACTGAACCAAACTTGCTTACTATCGACTTCGAGAAGGAT

GW forward 1

GW forward 2

VP2 forward 1

VP2 forward 2

VP2 internal 1

VP2 internal 2

VP2 internal 3 ACAGTATATTAACTGAACCAAACTTGCTTACTATCGACTTCGAGAAGGAT

VP2 internal 4 ACAGTATATTAACTGAACCAAACTTGCTTACTATCGACTTCGAGAAGGAT

VP2 reverse 1

VP2 reverse 2

GW reverse 1

GW reverse 2

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....
 BTV(10) -VP2 (ORF) GCCTATCTTGGTTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGACAA
 GW forward 1
 GW forward 2
 VP2 forward 1
 VP2 forward 2
 VP2 internal 1
 VP2 intrenal 2
 VP2 internal 3 GCCTATCTTGGTTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGACAA
 VP2 internal 4 GCCTATCTTGGTTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGACAA
 VP2 reverse 1
 VP2 reverse 2
 GW reverse 1
 GW reverse 2

1560 1570 1580 1590 1600

BTV (10) -VP2 (ORF) **ATGGATCAACTCGCGATGTTTAATGCCAAGCTTAAGATTGCGCGTGGCG**

GW forward 1 -----

GW forward 2 -----

VP2 forward 1 -----



VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

ATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTGGCG
ATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTGGCG

BTV (10) -VP2 (ORF)
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

1610 1620 1630 1640 1650
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
AGATCGCAACCCGGAAGGTGGATGACCCCTTGGAAACAATCGAGCGGTTCAT

AGATCGCAACCCGGAAGGTGGATGACCCCTTGGAAACAATCGAGCGGTTCAT
AGATCGCAACCCGGAAGGTGGATGACCCCTTGGAAACAATCGAGCGGTTCAT

BTV (10) -VP2 (ORF)
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

1660 1670 1680 1690 1700
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
GGGTACATTAAAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCCATA

GGGTACATTAAAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCCATA
GGGTACATTAAAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCCATA

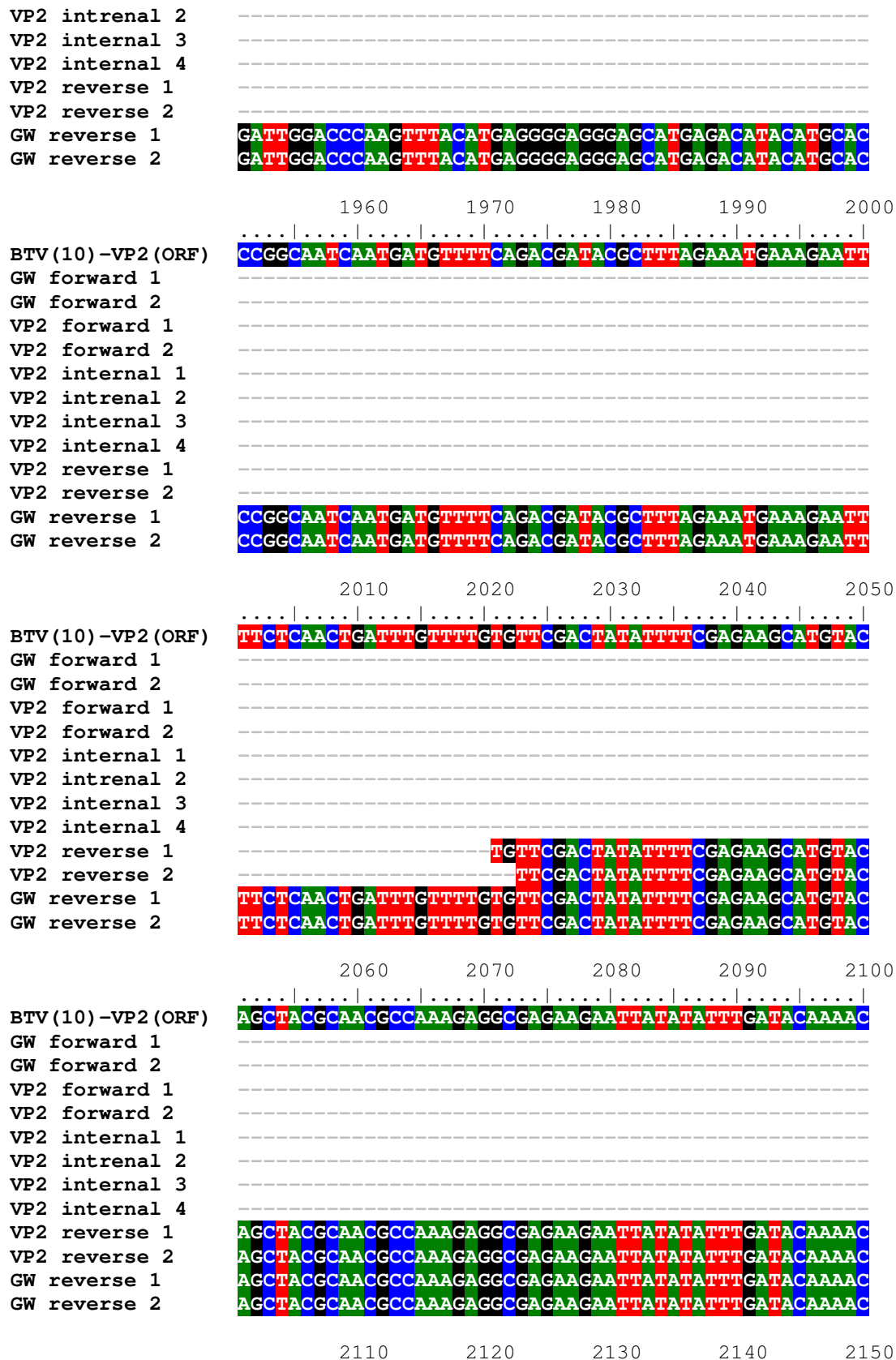
BTV (10) -VP2 (ORF)
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

1710 1720 1730 1740 1750
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
TTATGACCTACGTCTGCAATTATTTCGGCGACGCTCTCAGTCTGGAACAAA

TTATGACCTACGTCTGCAATTATTTCGGCGACGCTCTCAGTCTGGAACAAA
TTATGACCTACGTCTGCAATTATTTCGGCGACGCTCTCAGTCTGGAACAAA



	1760	1770	1780	1790	1800	
BTV (10) -VP2 (ORF)		AACAGTCAGCCGTATTCGAATATATGGCTCAACAAGATGATTTCCCCGCG				
GW forward 1						
GW forward 2						
VP2 forward 1						
VP2 forward 2						
VP2 internal 1						
VP2 intrenal 2						
VP2 internal 3	AACAGTCAGCCGTATTCGAATATATGGCTCAACAAGATGATTTCCCCGCG					
VP2 internal 4	AACAGTCAGCCGTATTCGAATATATGGCTCAACAAGATGATTTCCCCGCG					
VP2 reverse 1						
VP2 reverse 2						
GW reverse 1						
GW reverse 2						
	1810	1820	1830	1840	1850	
BTV (10) -VP2 (ORF)		CTAACAGATTATACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGGAAC				
GW forward 1						
GW forward 2						
VP2 forward 1						
VP2 forward 2						
VP2 internal 1						
VP2 intrenal 2						
VP2 internal 3	CTAACAGATTATACGAAGGAGAAGACGGTA					
VP2 internal 4	CTAACAGATTATACGAAGGAGAAGACGGTA					
VP2 reverse 1						
VP2 reverse 2						
GW reverse 1	TACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGGAAC					
GW reverse 2	TACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGGAAC					
	1860	1870	1880	1890	1900	
BTV (10) -VP2 (ORF)		CTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATGAAC				
GW forward 1						
GW forward 2						
VP2 forward 1						
VP2 forward 2						
VP2 internal 1						
VP2 intrenal 2						
VP2 internal 3						
VP2 internal 4						
VP2 reverse 1						
VP2 reverse 2						
GW reverse 1	CTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATGAAC					
GW reverse 2	CTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATGAAC					
	1910	1920	1930	1940	1950	
BTV (10) -VP2 (ORF)		GATTGGACCCAAGTTTACATGAGGGGAGGGAGCATGAGACATACATGCAC				
GW forward 1						
GW forward 2						
VP2 forward 1						
VP2 forward 2						
VP2 internal 1						





BTV (10) -VP2 (ORF)	ACGTCGGTGTGCACCGATTGGATATTTTACGCGAAACCTTCCCCAACTT
GW forward 1	
GW forward 2	
VP2 forward 1	
VP2 forward 2	
VP2 internal 1	
VP2 internal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	ACGTCTGGTGTGCACCGATTGGATATTTTACGCGAAACCTTCCCCAACTT
VP2 reverse 2	ACGTCTGGTGTGCACCGATTGGATATTTTACGCGAAACCTTCCCCAACTT
GW reverse 1	ACGTCTGGTGTGCACCGATTGGATATTTTACGCGAAACCTTCCCCAACTT
GW reverse 2	ACGTCTGGTGTGCACCGATTGGATATTTTACGCGAAACCTTCCCCAACTT
	2160 2170 2180 2190 2200
BTV (10) -VP2 (ORF)	TCTTAGGCACGTTATGAATTTACGCGATGTCAAACGCATATGCGATCTGA
GW forward 1	
GW forward 2	
VP2 forward 1	
VP2 forward 2	
VP2 internal 1	
VP2 internal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	TCTTAGGCACGTTATGAATTTACGCGATGTCAAACGCATATGCGATCTGA
VP2 reverse 2	TCTTAGGCACGTTATGAATTTACGCGATGTCAAACGCATATGCGATCTGA
GW reverse 1	TCTTAGGCACGTTATGAATTTACGCGATGTCAAACGCATATGCGATCTGA
GW reverse 2	TCTTAGGCACGTTATGAATTTACGCGATGTCAAACGCATATGCGATCTGA
	2210 2220 2230 2240 2250
BTV (10) -VP2 (ORF)	ATGTGATTAAATTTCTTTCCATTGTTATTTCTGATT CAGGATAATATTTCCG
GW forward 1	
GW forward 2	
VP2 forward 1	
VP2 forward 2	
VP2 internal 1	
VP2 internal 2	
VP2 internal 3	
VP2 internal 4	
VP2 reverse 1	ATGTGATTAAATTTCTTTCCATTGTTATTTCTGATT CAGGATAATATTTCCG
VP2 reverse 2	ATGTGATTAAATTTCTTTCCATTGTTATTTCTGATT CAGGATAATATTTCCG
GW reverse 1	ATGTGATTAAATTTCTTTCCATTGTTATTTCTGATT CAGGATAATATTTCCG
GW reverse 2	ATGTGATTAAATTTCTTTCCATTGTTATTTCTGATT CAGGATAATATTTCCG
	2260 2270 2280 2290 2300
BTV (10) -VP2 (ORF)	TATTGGCATAGACAGTGGTCGATCCCAATGATTTTGGTTGGAAGTCGT
GW forward 1	
GW forward 2	
VP2 forward 1	
VP2 forward 2	
VP2 internal 1	
VP2 internal 2	
VP2 internal 3	

```
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2
```

TATTGGCATAGACAGTGGTCGATCCCAATGATTTTGTTTGGTGAAGTCGT
 TATTGGCATAGACAGTGGTCGATCCCAATGATTTTGTTTGGTGAAGTCGT
 TATTGGCATAGACAGTGGTCGATCCCAATGATTTTGTTTGGTGAAGTCGT
 TATTGGCATAGACAGTGGTCGATCCCAATGATTTTGTTTGGTGAAGTCGT

2310 2320 2330 2340 2350

BTv (10) -VP2 (ORF)
 GW forward 1
 GW forward 2
 VP2 forward 1
 VP2 forward 2
 VP2 internal 1
 VP2 internal 2
 VP2 internal 3
 VP2 internal 4
 VP2 reverse 1
 VP2 reverse 2
 GW reverse 1
 GW reverse 2

CAGGCTAATCCCTATCGAAGTTGGGGCGTATGCAAAATAGATTTGGATTTA
 CAGGCTAATCCCTATCGAAGTTGGGGCGTATGCAAAATAGATTTGGATTTA
 CAGGCTAATCCCTATCGAAGTTGGGGCGTATGCAAAATAGATTTGGATTTA
 CAGGCTAATCCCTATCGAAGTTGGGGCGTATGCAAAATAGATTTGGATTTA

BTv (10) -VP2 (ORF)
 GW forward 1
 GW forward 2
 VP2 forward 1
 VP2 forward 2
 VP2 internal 1
 VP2 internal 2
 VP2 internal 3
 VP2 internal 4
 VP2 reverse 1
 VP2 reverse 2
 GW reverse 1
 GW reverse 2

[illegible]

BTv(10)-VP2(ORF)
GW forward 1
GW forward 2
VP2 forward 1
VP2 forward 2
VP2 internal 1
VP2 internal 2
VP2 internal 3
VP2 internal 4
VP2 reverse 1
VP2 reverse 2
GW reverse 1
GW reverse 2

2410 2420 2430 2440 2450

...|...|...|...|...|...|...|...|...|...|...|...|

CAGGATGCGGATGATACCCATAAAGAGTTTGGCTCAATATGTTTTGAGTA

CAGGATGCGGATGATACCCATAAAGAGTTTGGCTCAATATGTTTTGAGTA

CAGGATGCGGATGATACCCATAAAGAGTTTGGCTCAATATGTTTTGAGTA

CAGGATGCGGATGATACCCATAAAGAGTTTGGCTCAATATGTTTTGAGTA

CAGGATGCGGATGATACCCATAAAGAGTTTGGCTCAATATGTTTTGAGTA

BTV (10) -VP2 (ORF)

2460 2470 2480 2490 2500
TTTACTACGACAAAAATTTCTCAAGGTGAGATAGATGTTCCGGTCATCA



GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	TTATACTACGACAAAAATTTCTCAAGGTGAGATAGATGTTCCGGTCATCA
VP2 reverse 2	TTATACTACGACAAAAATTTCTCAAGGTGAGATAGATGTTCCGGTCATCA
GW reverse 1	TTATACTACGACAAAAATTTCTCAAGGTGAGATAGATGTTCCGGTCATCA
GW reverse 2	TTATACTACGACAAAAATTTCTCAAGGTGAGATAGATGTTCCGGTCATCA
	2510 2520 2530 2540 2550
BTV (10) -VP2 (ORF)	
GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	CGTCTAAATTAGACACACTTAAGTTGCATATAGCCTCTTTATGTGCGGGA
VP2 reverse 2	CGTCTAAATTAGACACACTTAAGTTGCATATAGCCTCTTTATGTGCGGGA
GW reverse 1	CGTCTAAATTAGACACACTTAAGTTGCATATAGCCTCTTTATGTGCGGGA
GW reverse 2	CGTCTAAATTAGACACACTTAAGTTGCATATAGCCTCTTTATGTGCGGGA
	2560 2570 2580 2590 2600
BTV (10) -VP2 (ORF)	
GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	CTAGCTGATTCTCTTGTGTATACGCTGCCTGTAGCGCATCCTAAGAAAAG
VP2 reverse 2	CTAGCTGATTCTCTTGTGTATACGCTGCCTGTAGCGCATCCTAAGAAAAG
GW reverse 1	CTAGCTGATTCTCTTGTGTATACGCTGCCTGTAGCGCATCCTAAGAAAAG
GW reverse 2	CTAGCTGATTCTCTTGTGTATACGCTGCCTGTAGCGCATCCTAAGAAAAG
	2610 2620 2630 2640 2650
BTV (10) -VP2 (ORF)	
GW forward 1	-----
GW forward 2	-----
VP2 forward 1	-----
VP2 forward 2	-----
VP2 internal 1	-----
VP2 internal 2	-----
VP2 internal 3	-----
VP2 internal 4	-----
VP2 reverse 1	TATCGTTCTCATTATTGTAGGGGATGATAAGCTTGAGCCACAAGTACGCT



```
VP2 forward 1 -----
VP2 forward 2 -----
VP2 internal 1 -----
VP2 internal 2 -----
VP2 internal 3 -----
VP2 internal 4 -----
VP2 reverse 1 GTAAAGTGGTATTGGTTAGGATGCCCGGGCATGTCTTTGGAAATGACGAG
VP2 reverse 2 GTAAAGTGGTATTGGTTAGGATGCCCGGGCATGTCTTTGGAAATGACGAG
GW reverse 1 -----
GW reverse 2 -----
```

```
                2860      2870      2880
.....|.....|.....|.....|.....|.....|.....|.....
BTV(10)-VP2(ORF) CTAATGACGAAGCTACTAAATGTTTAGAGATCT
GW forward 1 -----
GW forward 2 -----
VP2 forward 1 -----
VP2 forward 2 -----
VP2 internal 1 -----
VP2 internal 2 -----
VP2 internal 3 -----
VP2 internal 4 -----
VP2 reverse 1 CTAATGACGAAGCTACTAAATGTTTAGAGATCT
VP2 reverse 2 CTAATGACGAAGCTACTAAATGTTTAGAGATCT
GW reverse 1 -----
GW reverse 2 -----
```

APPENDIX B

Forward nucleotide sequence alignments of recombinant pET160-DEST constructs with BTV(10)-VP2(aa450), BTV(10)-VP2(aa650) and BTV(10)-VP2(ORF) reference sequence to verify insert orientation and reading frame with Lumio™ and polyhistidine fusion tags

	710	720	730	740	750
BTV(10) VP2 (aa450)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
T7 forward 1 (aa450)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
T7 forward 2 (aa450)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
BTV(10)-VP2 (aa650)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
T7 forward 1 (aa650)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
T7 forward 2 (aa650)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
BTV(10)-VP2 (ORF)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
T7 forward 1 (ORF)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				
T7 forward 2 (ORF)	AAACCTGGATTTATTACGAAGTTTAACAACGACAGAGATGTTCCACATCT				

	760	770	780	790	800
BTV(10) VP2 (aa450)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
T7 forward 1 (aa450)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
T7 forward 2 (aa450)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
BTV(10)-VP2 (aa650)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
T7 forward 1 (aa650)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
T7 forward 2 (aa650)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
BTV(10)-VP2 (ORF)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
T7 forward 1 (ORF)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				
T7 forward 2 (ORF)	TACAGGGAGCGGCTTATGCCTTAAAAATCAACGCATGAAC TAGTAGCTCAT				

	810	820	830	840	850
BTV(10) VP2 (aa450)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
T7 forward 1 (aa450)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
T7 forward 2 (aa450)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
BTV(10)-VP2 (aa650)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
T7 forward 1 (aa650)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
T7 forward 2 (aa650)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
BTV(10)-VP2 (ORF)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
T7 forward 1 (ORF)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				
T7 forward 2 (ORF)	TCCGAAAGGGAAAGCAGCAGCGAGACTTATCAGGTTGGGACGCAGAGATG				

	860	870	880	890	900
BTV(10) VP2 (aa450)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
T7 forward 1 (aa450)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
T7 forward 2 (aa450)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
BTV(10)-VP2 (aa650)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
T7 forward 1 (aa650)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
T7 forward 2 (aa650)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
BTV(10)-VP2 (ORF)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
T7 forward 1 (ORF)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				
T7 forward 2 (ORF)	GATACAGCTGCGTAAAGGTACAAAGATTGGGTATAGAGGGCAGCCGTACG				

	910	920	930	940	950
BTV(10) VP2 (aa450)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA				



T7 forward 1 (aa450)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
T7 forward 2 (aa450)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
BTV(10)-VP2 (aa650)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
T7 forward 1 (aa650)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
T7 forward 2 (aa650)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
BTV(10)-VP2 (ORF)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
T7 forward 1 (ORF)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
T7 forward 2 (ORF)	AACGATTTCATATCCAGCTTAGTTCAAGTAATCATTAAAGGAAGAGTTCCA
	960 970 980 990 1000
BTV(10) VP2 (aa450)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
T7 forward 1 (aa450)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
T7 forward 2 (aa450)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
BTV(10)-VP2 (aa650)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
T7 forward 1 (aa650)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
T7 forward 2 (aa650)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
BTV(10)-VP2 (ORF)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
T7 forward 1 (ORF)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
T7 forward 2 (ORF)	GATGAGATTAGAGACGAAATCGCTGAACCTTAATAGGATTAAAGATGAATG
	1010 1020 1030 1040 1050
BTV(10) VP2 (aa450)	GAAAAACGCCGCGTATGATAGAACTAAAAACGCCGCAATTGGAGCTCTGCA
T7 forward 1 (aa450)	G
T7 forward 2 (aa450)	G
BTV(10)-VP2 (aa650)	GAAAAACGCCGCGTATGATAGAACTAAAAACGCCGCAATTGGAGCTCTGCA
T7 forward 1 (aa650)	G
T7 forward 2 (aa650)	G
BTV(10)-VP2 (ORF)	GAAAAACGCCGCGTATGATAGAACTAAAAACGCCGCAATTGGAGCTCTGCA
T7 forward 1 (ORF)	G
T7 forward 2 (ORF)	G
	1060 1070 1080 1090 1100
BTV(10) VP2 (aa450)	AAATACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCA
T7 forward 1 (aa450)	
T7 forward 2 (aa450)	
BTV(10)-VP2 (aa650)	AAATACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCA
T7 forward 1 (aa650)	
T7 forward 2 (aa650)	
BTV(10)-VP2 (ORF)	AAATACTCTCGGCGATCGGACGTAAAAATGTTGGATGTTCAAGAGGAGCCA
T7 forward 1 (ORF)	
T7 forward 2 (ORF)	
	1110 1120 1130 1140 1150
BTV(10) VP2 (aa450)	AAGGATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAA
T7 forward 1 (aa450)	
T7 forward 2 (aa450)	
BTV(10)-VP2 (aa650)	AAGGATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAA
T7 forward 1 (aa650)	
T7 forward 2 (aa650)	
BTV(10)-VP2 (ORF)	AAGGATGAGATGGCACTATCCACTAGGTTTCAGTTCAAATTTGGACGAGAA
T7 forward 1 (ORF)	
T7 forward 2 (ORF)	



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                                1160      1170      1180      1190      1200
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV (10) VP2 (aa450)  ATTCATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTC
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) ATTCATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTC
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF)   ATTCATTAGAACCGATCAAGAGCATGTAAACATATTTGAAGTAGGTGGTC
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                                1210      1220      1230      1240      1250
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV (10) VP2 (aa450)  CCGCAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACC
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) CCGCAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACC
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF)   CCGCAACAGATGATGGGAGGTTTTACGCGCTGATCGCTATAGCGGCTACC
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                                1260      1270      1280      1290      1300
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV (10) VP2 (aa450)  GATACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACG
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) GATACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACG
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF)   GATACACAACAAGGTCGCGTGTGGAGGACGAATCCTTATCCGTGTCTACG
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                                1310      1320      1330      1340      1350
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV (10) VP2 (aa450)  CGGTGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACAT
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) CGGTGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACAT
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF)   CGGTGCGCTAATTGCTGCTGAGTGTGAGCTTGGTGACGTGTACTTCACAT
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                                1360      1370      1380      1390      1400
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
BTV (10) VP2 (aa450)  TCGGCCAAACATATAAGTGGAGTTTGCGTCCAGAATATGGTCAACGTGAG
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) TCGGCCAAACATATAAGTGGAGTTTGCGTCCAGAATATGGTCAACGTGAG
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF)   TCGGCCAAACATATAAGTGGAGTTTGCGTCCAGAATATGGTCAACGTGAG
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T7 forward 1 (ORF)

T7 forward 2 (ORF)

BTV (10) VP2 (aa450)

T7 forward 1 (aa450)

T7 forward 2 (aa450)

BTV (10) -VP2 (aa650)

T7 forward 1 (aa650)

T7 forward 2 (aa650)

BTV (10) -VP2 (ORF)

T7 forward 1 (ORF)

T7 forward 2 (ORF)

BTV (10) VP2 (aa450)

T7 forward 1 (aa450)

T7 forward 2 (aa450)

BTV (10) -VP2 (aa650)

T7 forward 1 (aa650)

T7 forward 2 (aa650)

BTV (10) -VP2 (ORF)

T7 forward 1 (ORF)

T7 forward 2 (ORF)

BTV (10) VP2 (aa450)

T7 forward 1 (aa450)

T7 forward 2 (aa450)

BTV (10) -VP2 (aa650)

T7 forward 1 (aa650)

T7 forward 2 (aa650)

BTV (10) -VP2 (ORF)

T7 forward 1 (ORF)

T7 forward 2 (ORF)

BTV (10) VP2 (aa450)

T7 forward 1 (aa450)

T7 forward 2 (aa450)

BTV (10) -VP2 (aa650)

T7 forward 1 (aa650)

T7 forward 2 (aa650)

BTV (10) -VP2 (ORF)

T7 forward 1 (ORF)

T7 forward 2 (ORF)

BTV (10) VP2 (aa450)

T7 forward 1 (aa450)

T7 forward 2 (aa450)

BTV (10) -VP2 (aa650)



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T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF)  GATGTTTGATCGTATGATTCAAGGCGGATGGGATCAGGAGCGATTTAAAC
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

                                1660      1670      1680      1690      1700
                                ....|....|....|....|....|....|....|....|....|
BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) TGCACAGTATATTAACCTGAACCAAACCTGCTTACTATCGACTTCGAGAAG
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF)  TGCACAGTATATTAACCTGAACCAAACCTGCTTACTATCGACTTCGAGAAG
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

                                1710      1720      1730      1740      1750
                                ....|....|....|....|....|....|....|....|....|
BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) GATGCCATCTTGGTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGA
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF)  GATGCCATCTTGGTTCGCGCTCTGAATTAGTATTTCCACCTTATTATGA
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

                                1760      1770      1780      1790      1800
                                ....|....|....|....|....|....|....|....|....|
BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) CAAATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTG
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF)  CAAATGGATCAACTCGCCGATGTTTAATGCCAAGCTTAAGATTGCGCGTG
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

                                1810      1820      1830      1840      1850
                                ....|....|....|....|....|....|....|....|....|
BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) GCGAGATCGCAACCCGGAAGGTGGATGACCCCTTGAACAATCGAGCGGTT
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF)  GCGAGATCGCAACCCGGAAGGTGGATGACCCCTTGAACAATCGAGCGGTT
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

                                1860      1870      1880      1890      1900
                                ....|....|....|....|....|....|....|....|....|
BTV(10)VP2 (aa450) -----

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T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) CATGGGTACATTAAAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCC
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) CATGGGTACATTAAAAACGTCAGCGGAGTCTCTAGGGTATGTGTTAGGGCC
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

1910 1920 1930 1940 1950
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10) VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) ATATTATGACCTACGTCTGCAATTATTTCGGCGACGCTCTCAGTCTTGAAC
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) ATATTATGACCTACGTCTGCAATTATTTCGGCGACGCTCTCAGTCTTGAAC
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

1960 1970 1980 1990 2000
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10) VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) AAAAAACAGTCAGCCGTATTTCGAATATATGGCTCAACAAGATGATTTCCCC
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) AAAAAACAGTCAGCCGTATTTCGAATATATGGCTCAACAAGATGATTTCCCC
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2010 2020 2030 2040 2050
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10) VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) GCGCTAACAGATTATACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGG
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) GCGCTAACAGATTATACGAAGGAGAAGACGGTATGCCCTCATTTCAGGCGG
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2060 2070 2080 2090 2100
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10) VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) AACCTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATG
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) AACCTTTTACACTTTCCGCAAGGTAGCGTTGATCATTTTATCGAGTTATG
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----



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                2110      2120      2130      2140      2150
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) VP2 (aa450)
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) AACGATTGGACCCAAGTTTACATGAGGGGAGGGAGCATGAGACATACATG
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF) AACGATTGGACCCAAGTTTACATGAGGGGAGGGAGCATGAGACATACATG
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                2160      2170      2180      2190      2200
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) VP2 (aa450)
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650) CACCCGGCAATCAATGATTAGTCTAGA
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF) CACCCGGCAATCAATGATGTTTTTCAGACGATACGCTTTTAGAAATGAAAGA
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                2210      2220      2230      2240      2250
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) VP2 (aa450)
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650)
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF) ATTTTCTCAACTGATTTGTTTTGTGTTCTGACTATATTTTTCGAGAAGCATG
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                2260      2270      2280      2290      2300
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) VP2 (aa450)
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650)
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF) TACAGCTACGCAACGCCAAAGAGGCGAGAAGAAATTATATATTTGATACAA
T7 forward 1 (ORF)
T7 forward 2 (ORF)

                2310      2320      2330      2340      2350
      ....|....|....|....|....|....|....|....|....|....|
BTV (10) VP2 (aa450)
T7 forward 1 (aa450)
T7 forward 2 (aa450)
BTV (10)-VP2 (aa650)
T7 forward 1 (aa650)
T7 forward 2 (aa650)
BTV (10)-VP2 (ORF) AACACGTCGGGTGTGCACCGATTGGATATTTTACGCGAAACCTTCCCCAA
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T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2360 2370 2380 2390 2400
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **CTTTCCTTAGGCACGTTATGAATTTACGCGATGTCAAACGCATATGCGATC**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2410 2420 2430 2440 2450
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **TGAATGTGATTAATTTCTTTCATTGTTATTTCTGATTCCAGGATAATATT**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2460 2470 2480 2490 2500
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **TGGTATTGGCATAGACAGTGGTCGATCCCAATGATTTTCTTTGGTGAAGT**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2510 2520 2530 2540 2550
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **CGTCAGGCTAATCCCTATCGAAGTTGGGGCGTATGCAAAATAGATTTGGAT**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2560 2570 2580 2590 2600
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----



T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) TTAAGAGCTTCCTCAATTTTCATTAGGTTTCACCCCGGCGACTCAAAGAAG
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2610 2620 2630 2640 2650
....|....|....|....|....|....|....|....|....|....|

BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) AAGCAGGATGCGGATGATACCCATAAAGAGTTTGGCTCAATATGTTTTGA
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2660 2670 2680 2690 2700
....|....|....|....|....|....|....|....|....|....|

BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) GTATTATACTACGACAAAAATTTCTCAAGGTGAGATAGATGTTCCGGTCA
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2710 2720 2730 2740 2750
....|....|....|....|....|....|....|....|....|....|

BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) TCACATCTAAATTAGACACACTTAAGTTGCATATAGCCTCTTTATGTGCG
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2760 2770 2780 2790 2800
....|....|....|....|....|....|....|....|....|....|

BTV(10)VP2 (aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2 (aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2 (ORF) GGACTAGCTGATTCTCTTGTGTATACGCTGCCTGTAGCGCATCCTAAGAA
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2810 2820 2830 2840 2850
....|....|....|....|....|....|....|....|....|....|

BTV(10)VP2 (aa450) -----



T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **AAGTATCGTTCTCATTATTGTAGGGGATGATAAGCTTGAGCCACAAGTAC**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2860 2870 2880 2890 2900
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **GCTCAGAGCAAATCGTAAATAAGTATCATTATTCGCGGAGGCATATCTCA**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2910 2920 2930 2940 2950
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **GGGGTCGTTTCGATCTCGTGACAGAACGGCCAACGGAAGGTACATTC**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

2960 2970 2980 2990 3000
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **TATGGGGATTACGAGACATCGAATATGTGATAAATCGATATTGAAATATA**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----

3010 3020 3030 3040 3050
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

BTV(10)VP2(aa450) -----
T7 forward 1 (aa450) -----
T7 forward 2 (aa450) -----
BTV(10)-VP2(aa650) -----
T7 forward 1 (aa650) -----
T7 forward 2 (aa650) -----
BTV(10)-VP2(ORF) **AATGTAAAGTGGTATTGGTTAGGATGCCCGGCATGCTTTGGAAATGAC**
T7 forward 1 (ORF) -----
T7 forward 2 (ORF) -----



	3060	3070	3080	3090	3100
					
BTV(10)VP2(aa450)	-----				
T7 forward 1 (aa450)	-----				
T7 forward 2 (aa450)	-----				
BTV(10)-VP2(aa650)	-----				
T7 forward 1 (aa650)	-----				
T7 forward 2 (aa650)	-----				
BTV(10)-VP2(ORF)	GAGCTAATGACGAAGCTACTAAATGTTTAGAGATCT				
T7 forward 1 (ORF)	-----				
T7 forward 2 (ORF)	-----				