Next generation vaccine for bluetongue virus serotype-8 and the neutralizing immune response in a mouse model

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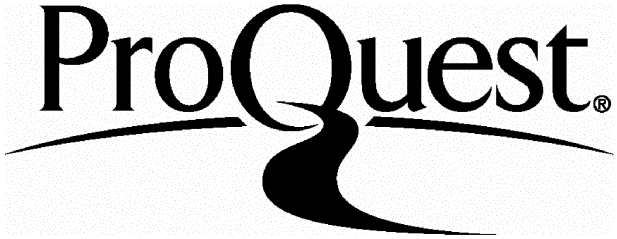

Declaration

I declare that all of the work submitted herewith has been carried out by myself.
Collaborative work is acknowledged where present.

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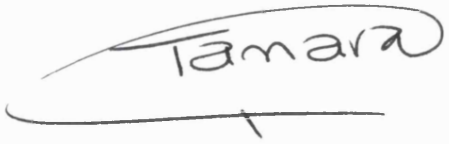

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I. ABSTRACT

The recent emergence of BT in Europe and the spread of BTV-8 further north in the region than ever before, has emphasised the importance of vaccination against this economically important pathogen. The chemically inactivated and live attenuated BTV vaccines that are currently available do provide significant levels of protection (Savini et al., 2007). However, there are safety concerns over both vaccines, and it has not been possible to 'distinguish infected from vaccinated animals' (DIVA assays), making surveillance more difficult. A new generation of BTV vaccines is therefore required for use as part of appropriate surveillance and control strategies.

'Next generation' BTV-8 subunit-vaccine-candidates were prepared as: individual bacterial-expressed viral-proteins; 'DNA vaccines' composed of plasmid DNA carrying BTV genes; and recombinant Modified Vaccinia Ankara (rMVA) also carrying BTV genes. These systems were used to explore the potential of BTV VP2-fragment-1, -2 and -3, VP2-complete, VP5 and VP7 as subunit vaccines.

Different vaccination strategies were evaluated in IFNAR -/- mice: by vaccination with bacterial expressed proteins (fragmented VP2+ VP5 + VP7; or complete VP2+ VP5 + VP7) combined with Montanide, in a prime-boost regime –administered at a three week interval. A second vaccination strategy was based on (in situ) expression of viral proteins, by priming with plasmid DNA containing cDNA copies of BTV VP2, VP5 and/or VP7 capsid-genes, followed by vaccination with recombinant Modified Vaccinia Ankara (rMVA) expressing the same proteins, at a three week interval. An alternative prime-boost regime was also used, vaccinating (prime and boost) with rMVA (expressing these proteins) on both occasions.

The unvaccinated-control mice, as well as those vaccinated with VP7 (alone) or with (fragmented VP2+ VP5 + VP7) were not protected against a subsequent challenge with a lethal dose (10pfu) of BTV-8. However, 50% of mice vaccinated with complete VP2+ VP5 + VP7 were protected.

All of the mice vaccinated with DNA-rMVA or rMVA-rMVA expressing VP2; or VP2, VP5 and VP7, were protected, with VP2-alone generating the highest level of protection. Further work will be needed to test different combinations of these BTV-subunit vaccine candidates, to validate their use and efficacy in ruminants (the natural hosts for BTV infection), and further investigate their potential for protection against heterologous serotypes.

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Science without religion is lame, religion without science is blind.

Fool me once, shame on you. Fool me twice, shame on me!

When the character of a man is not clear to you, look at his friends.

When the power of love overcomes the love of power, the world will know peace

Never tell your problems to anyone...20% don't care and the other 80% are glad you have them. - Lou Holtz

Forgive your enemies, but never, never forget their names. John F. Kennedy

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VI. LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
ADCC	antibody-dependant cellular cytotoxicity
AGE	agarose gel electrophoresis
AHSV	African horse sickness virus
AMV-RT	Avian myeloblastosis virus-reverse transcriptase
APC	antigen presenting cells
APS	ammonium persulphate
ATP	adenosine triphosphate
BCR	B cell receptors
β-ME	β-mercaptoethanol
BHK	baby hamster kidney
bp	base pair (s)
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
BT	bluetongue
BTV	Bluetongue Virus
C.	Culicoides
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CLP	core-like particle
CMI	cell-mediated immunity
CMV	cytomegalovirus
CO2	carbon dioxide
CPE	cytopathic effect
CpG	cytosine phosphate guanosine
CSU	central services unit
Ct	threshold cycle
CTL	cytotoxic T lymphocyte
Da	Dalton
DAPI	4‟6‟-diamidino-2-phenylindole
DC	dendritic cells
DIVA	differentiation infected vs. vaccinated animals
DMEM	dulbecco's modified Eagle's medium
DMSO	dimethyl Sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPI	days post infection
ds	double stranded
dsRNA	double stranded ribonucleic acid
DTT	dithiotheitol
EDTA	ethylenediaminetetraacetic acid
EEV	Equine encephalosis virus
EDTA	ethylene diamine tetra acetate
EHDV	Epizootic haemorrhagic disease virus
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
EtBr	ethidium bromide
FCS	foetal calf serum
G	gravity or gravities

'g'	grams
GMEM	Glasgows modified eagles medium
GST	glutathione-s-transferase
HRP	horse radish peroxidise
Hour	h
IAH	Institute for Animal Health
IFN	interferon
I/m	intramascular
I/v	intravenous
IPTG	isopropylthio-β-D-galactoside
ISVP	infectious subviral particle
kDa	kilodalton
l	litre
LB-medium	Luria-Bertani medium
LMP	low melting point
μ	micro
m	milli
M	molar
Ma	milliamp
Min	minutes
MOI	multiplicity of infection
MMLV	Moloney murine leukemia virus
mRNA	messenger RNA
n	nano
NS	non-structural
nt	nucleotide
NTP	nucleotide triphosphate
ORF	open reading frame
PABP	poly(A) binding protein
PBMC	peripheral blood mononuclear cells
O.I.E	Office International des Epizootics
OVI	Onderstepoort Veterinary Research Institute
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu	plaque forming unit
PKR	protein kinase R
RNA	ribonucleic acid
RT	reverse-transcription
RT-PCR	reverse transcription-polymerase chain reaction
Seg-	segment
SAP	shrimp alkaline phosphatase
S	second
S/C	subcutaneous
SDS	sodium dodecyl sulphate
SPAT	single Primer Amplification technique
ssRNA	single-stranded RNA
SSII RT	superscript II reverse transcriptase
SVP	subviral particle
TAE	tris-Acetate-EDTA
TCID50	tissue culture infective doses 50
TCR	trichloroacetic acid

TCR	T cell receptor
TE	tris-Acetate
TEMED	N, N, N', N, tetramethylethylenediamine
Th	T-helper cells
TK	thymidine kinase
TK^-	thymidine kinase negative
TLR	toll-like receptor
TOV	Toggenburg orbivirus
Tris	tris (hydroxymethyl) amino ethane
U	units
VLP	virus like particles
VNT	virus neutralisation test
VIB	virus inclusion body
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER 1
Introduction

1 Introduction

1.1 : Bluetongue

Bluetongue (BT) is a non-contagious arthropod born viral disease that is transmitted via the bites of adult females from certain species of *Culicoides* midges (Diptera-Ceratopogonidae) (Takamatsu et al., 2003; Boone et al., 2007; Darpel et al., 2007). The bluetongue virus (BTV) is occasionally also transmitted 'vertically' across the placenta, or in seminal fluid, or by an oral route (de Clercq et al 2008; Menzies et al 2008).

Although BTV can infect members of most ruminant species, as well as some predatory carnivores (Alexander et al., 1994; Schwartz-Cornil et al., 2008; Mertens and Diprose 2004; Breard et al., 2007), clinical signs of BT are most commonly observed in naïve sheep or some species of deer and can cause fatality levels >50% as in Cyprus in 1943 (Polydorou, 1978). Although cattle represent an epidemiologically important route of BTV spread and a virus reservoir, infection of cattle and goats is often inapparent, or causes only mild clinical signs. However during the northern European outbreak caused by BTV-8 (2006-2008), clinical signs developed in ~10% of cattle in affected areas with a case fatality rate up to10% in these animals (~1% of the total cattle population) (Shaw et al., 2007)

1.2 : Bluetongue virus

Bluetongue virus (BTV) is a member of the genus *Orbivirus* within the family *Reoviridae*, (Cowley and Gorman 1987; Aguero et al., 2002; Mertens et al., 2005; Forzan et al., 2007). The specificity of interactions between BTV outer capsid proteins and neutralising antibodies, determines the identity of the 26 known BTV serotypes (Roy, 1992a; Maan et al., 2007a, 2011, 2012; Anthony et al., 2007; Hofman et al., 2008). The genus *Orbivirus* also includes 21 other established virus species / serogroups (most of which also contain multiple virus strains / serotypes) as well as several virus strains / isolates that have not yet been characterised or assigned to recognised species (Mertens et al., 2005; Monaco et al., 2006; Aguero et al., 2002).

The icosahedral BTV particle has icosahedral symmetry, with three distinct capsid layers, including the sub-core, outer-core and outer-capsid layers, and is non-enveloped, (Grimes et al., 1998; Roy, 2003; Prasad et al., 1992; Mertens et al., 2005;

Forzan et al., 2007; Shaw et al., 2007). The BTV genome is composed of ten linear segments of dsRNA, most of which code for one viral protein. Seven structural proteins [VP1-VP7] are components of the BT virus-particle and four [NS1, NS2, NS3/NS3a and NS4] are non-structural (Mertens et al., 1984, Roy et al., 1990; Bonneau et al., 2001; Anthony et al., 2007, Belhouchet et al., 2011).

The BTV outer capsid is composed of two major structural proteins, VP2 (110-KDa) and VP5 (60 KDa) (encoded by genome segments 2 and 6 [Seg-2 and Seg-6] respectively, which are involved in cell attachment and virus entry. VP2 is the most variable of the BTV proteins, containing the major neutralisation-epitopes that are the determinants of BTV serotype (Mertens et al 1989; Roy, 1992a; Maan et al., 2007a; Calvo-Pinilla et al , 2009, de Maula et al 2000). VP5, which is the the second most variable of the BTV proteins influences virus neutralisation through its conformational interaction with VP2 (Maan et al., 2008, Mertens et al 1989, deMaula et 2000). The outer capsid proteins may also be associated with small amounts of one of the non-structural proteins (NS2)(Wade-Evans et al., 1996).

The BTV outer-capsid layer encloses the core-particle, which itself contains two protein layers (the core-surface and sub-core layers), which are composed of major structural proteins VP3 and VP7 (encoded by Seg-3 and Seg-7) respectively. VP3 and VP7 are conserved proteins, showing serological cross-reactions between all of the different BTV strains. The sub-core surrounds the virus genome, as well as three further minor proteins, VP1, VP4, and VP6, (encoded by Seg-1, 4, and 9 respectively). Four distinct, conserved non-structural proteins are also produced during the virus replication cycle (NS1, NS2, NS3/NS3A and NS4) encoded by Seg-6, 8, 10 and 9 respectively).

Like other members of the family *Reoviridae,* the BTV genome is composed of multiple (ten) linear segments of dsRNA (http://www.reoviridae.org/dsRNA_virus_proteins/BTV.htm). The BTV genome is approximately 19200 bp long, with segment ranging between 3,954 bp and 822 bp (Mertens et al 2005; Schwartz-Cornil et al., 2008). The BTV genome segments migrate in a characteristic pattern during 1% agarose gel electrophoresis (AGE), with three size ranges designated L (segments 1- 3), M (segments 4-6) and S (segments 7-10), generating a 3-3-4 pattern (Attoui et al., 2001; Huismans et al., 1983; French and Roy, 1990; Breard et al., 2003). The BTV genome is packaged as exactly one copy of each

dsRNA segment within the central space of the icosahedra core particle, (Gouet et al 1999 Roy et al., 1990; Prasad et al., 1992; Hassan et al., 2001; Bread et al., 2003) (Figure 1.1).

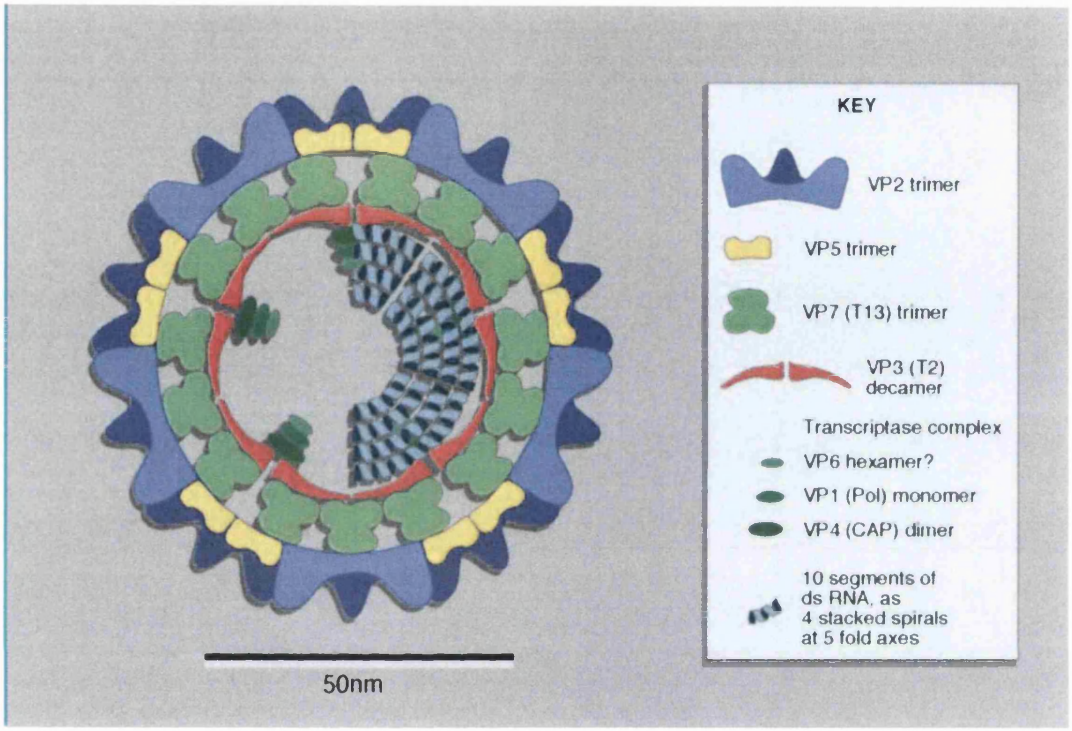


Figure 1-1 Schematic representation of the structure of the Bluetongue. Reproduced with permission Mertens, P. P. C., Maan, S., Samuel, A. & Attoui, H. (2005). *Orbivirus, Reoviridae.* In *Virus Taxonomy, VIIIth Report of the International Committee for the Taxonomy of Viruses (ICTV).*

1.3 BTV structural proteins

1.3.1 : Outer capsid proteins

a) VP2

Sixty trimers of VP2, form "triskelion" motifs (three interlocked spirals) on the outer-surface of the BTV particle (Hewat et al, 1994; Schwartz-Cornil et al., 2008). Cryo-electron microscopy (Cryo-EM) studies have shown that VP2 is the most exposed virion protein, with a protruding spike like structure (Hewat et al 1994, Forzan et al., 2007). The nucleotide sequences of VP2 show a maximum of ~30% nucleotide variation within the same serotype, with 29% (BTV-8 and BTV-18) to 59% (BTV-16 and BTV-22) variation within the major 'eastern' or 'western' geographic groups (VP2 topotypes) (Maan et al., 2007a).

VP2 is responsible for receptor binding, adsorption and entry of mammalian cells, resulting in delivery of the transcriptional active BTV core into the target cell cytoplasm (Forzan et al.,2007; Breard et al., 2003; Bonneau et al., 2001). The adsorption of virus, and penetration of the cell surface can occur within ten minutes of infecton, by clathrin-mediated endocytosis (Ross-Smith et al., 2008; Forzan et al., 2007). However, BTV can also use other clathrin independent cell entry pathways (Gold et al 2010). VP7 (which forms the surface layer of the BTV core) can also mediate cell attachment and penetration of insect cells by core particles (Mertens et al., 1996).

VP2 is responsible for BTV haemagglutination, which is specific for erythrocytes of certain vertebrate species and may be involved in persistence of the virus in the blood of the mammalian host (Hassan and Roy, 1999; Bonneau et al., 2001; Cowley and Gorman, 1987). It has been suggested that BTV haemagglutination activity, may help to stimulate transmission of BTV to *Culicoides* vectors during 'blood' feeding from the vertebrate host. The binding of BTV to erythrocytes reflects a strong affinity of VP2 for glycophorin A, which is a sialoglycoprotein component present on the erythrocyte surface membrane (Schwartz-Cornil et al., 2008).

b) VP5

The smaller outer capsid component VP5, is the second most variable of the BTV proteins (after VP2) (Maan et al., 2008). Like VP2, VP5 shows variations that correlate with virus serotype (although for VP5 this is not an absolute relationship and there are exceptions that do not fit the pattern). There are also variations in VP5, within each serotype that reflect the geographic origin of the virus isolate (VP5–topotypes) (Roy, 1992a; Schwartz-Cornil et al., 2008; Singh et al., 2004).

120 trimers of VP5 form globular motifs on the BTV outer surface (Hewat et al 1994; Schwartz-Cornil et al., 2008). Although VP5 is located in the outer capsid, it does not appear to have any distinct neutralising activity when used by itself, to raise antisera (Roy, 1992a; Huismans et al., 1983). However, VP5 is believed to enhance the generation of protective neutralisation activity of antibodies raised to VP2 alone. VP2-VP5 not only protected vaccinated sheep, but also elicited a higher neutralising antibody response (Roy et al., 1990). It has been suggested that interactions between VP5 and VP2, can affect the conformation of VP2 and consequently it's serological properties (Mertens et al., 1989; Cowley and Gorman 1989; DeMaula et al., 2000; Roy, 1992a).

VP5 can permeabilize the mammalian and *Culicoides* insect cell membrane, causing syncitium formation and cytotoxicity (Hassan et al 2001; Schwartz-Cornil et al., 2008). This reflects the high level of helical content in the amino terminus of VP5, with a strongly predicted 'coiled–coil' structure that resembles that of membrane fusion proteins (Hassan et al., 2001). Uncoating of the virus particle requires an initial low-pH step within the endosome (Noad and Roy, 2009; Schwartz-Cornil et al., 2008; Hassan et al., 2001; Hutchinson, 1999). This may help to trigger VP2 degradation and expose a functional form of VP5. The structurally altered VP5 induces destabilization of the endosomal membrane and release of the core into the cytosol (Schwartz-Cornil et al., 2008; Hassan et al., 2001; Noad and Roy, 2009; Forzan et al., 2007).

1.3.2 : Core proteins

a) Major structural proteins VP7 and VP3

The BTV core contains two major structural-proteins, VP3 and VP7. The core-surface layer is composed of 780 copies of VP7 arranged as 260 trimers, arranged with T=13 icosahedral symmetry. The inner sub-core layer is composed of 120 copies of VP3 arranged with T=2 quasi icosahedral symmetry (Grimes et al 1998; Roy, 2003). VP7 is encoded by a Seg-7 and has a Mr of 38KDa, while VP3 with a Mr of 100KDa, is encoded by Seg-3. Both proteins are extremely hydrophobic (Prasad et al., 1992; Roy, 1992a; Maan et al., 2008).

Although BTV VP3 is very highly conserved between different BTV isolates, VP7 is encoded by the third least conserved of the BTV genome segments (Seg-7) (Maan et al., 2008). However, both VP3 and VP7, show serological cross-reactions between different BTV strains, involving antibodies (particularly those against VP7) that can be detected in BTV serogroup–specific serological assays. These include competitive enzyme linked immunosorbent assay (c-ELISA), which are used as diagnostic tests to detect BTV specific antibodies and confirm BTV infection. RT-PCR assays targeting these or other'conserved' genome segments can also be used to identify / detect any bluetongue virus isolate (Anthony et al., 2007; OIE, 2004; Shaw et al 2007).

Sheep vaccinated with a recombinant capripox virus containing the VP7 gene of BTV-1, were partially protected when challenged with virulent strains of BTV-1 or BTV-3 (Wade-Evans et al., 1996). This protection was achieved in the absence of neutralising antibodies to either the homologous or the heterologous BTV serotypes, suggesting the involvement of a protective cell mediated response targeting VP7.

VP3 and VP7 play an important role in maintaining the integrity of the BTV core structure (French and Roy, 1990, Grimes et al., 1998). This protects the dsRNA virus genome from cellular antiviral surveillance and therefore helps to prevent activation of type I interferon production, or RNA silencing mechanisms (Noad and Roy, 2009; Schwartz-Cornil et al., 2008). While BTV cores have only low infectivity for mammalian cells, they are highly infectious for both adult *Culicoides* midges and *Culicoides* sonorensis derived KC cells (Mertens et al., 1996). This demonstrates that VP7 can mediate attachment and penetration of insect cells in the absence of VP2 and VP5. This binding may be facilitated through an arginine - glycine - aspartate (RGD) tripeptide motif that is present and conserved on the outer surface of each monomer of VP7 on the core surface (Ross-Smith et al., 2008).

b) Minor structural proteins (VP1, VP4 and VP6)

VP1(Pol) , VP4(Cap) and VP6(Hel) are encoded by Seg-1, 4 and 9 respectively and are situated within the internal region of the core. These enzyme proteins are responsible for transcription, capping and unwinding of the viral mRNA respectively (Noad and Roy, 2009; Roy et al., 1990; Anthony et al., 2007; Perrin et al., 2007).

VP1(Pol): is the largest BTV protein with a Mr of (105KDa). It is a minor component of the BTV core and is encoded by the largest dsRNA segment (Seg-1) (Roy 1992a). VP1 is the viral RNA dependent RNA polymerase and is resposible not only for the synthesis of BTV mRNAs - using the viral genome segments as template (transcriptase), it also acts as a replicase - synthesising negative strands using mRNAs as templates, thereby reforming the dsRNA segments of progeny virus particles (Mertens and Diprose 2004; Schwartz-Cornil et al., 2008; Anthony et al., 2007). Seg-1 represents one of the most conserved regions of the BTV genome, and has provided a target suitable for real-time RT-PCR assays to detect any of the 26 BTV serotypes, as well as different strains of BTV representing both Eastern and Western topotypes (Shaw et al., 2007; Anthony et al., 2007).

BTV VP1 has optimal polymerase activity between 27°C to 37°C. This allows efficient replication of the virus in both insect and mammalian cells (Schwartz-Cornil et al., 2008). The active temperature range of the BTV polymerase is thought to play a central role in determination of the efficiency of BTV transmission by vector *Culicoides* at different temperatures. Consequently ambient temperature represents one of the most

important climatic variables that influence, both the seasonal and geographic distribution of BTV transmission and consequently the distribution of disease outbreaks.

VP4(CaP): The 5' ends of BTV mRNAs are capped and methylated during transcription. This stabilises the mRNAs and promotes their efficient translation, by the host cell translation apparatus. VP4 is a minor component of the BTV core and is the BTV capping enzyme. It also has nucleotide phosphohydrolase, guanylyltransferase and two distinct transmethylase activities, which allow it to synthesise type 1 Cap structures on the 5' end of the viral mRNAs (Ramadevi et al 1998; Roy, 1992a; Schwartz-Cornil et al., 2008).

VP6(Hel): has NTPase and helicase activity, allowing it to unwind duplex dsRNAs. It has been suggested that this activity separates the 5' end of the +ve and 3' end of –ve strands of the BTV genome segments, allowing VP1 to initiate mRNA synthesis during BTV replication (Roy, 1992a; Schwartz-Cornil et al., 2008). The BTV polymerase has a fully conservative mode of action. The helicase activity of VP6 (Hel) may therefore also mediate separation of the nascent +ve RNA chain from the –ve strand template, in order to allow the original +ve and -ve strands template to reanneal and reform the original dsRNA template.

1.3.3 Non structural proteins (NS1, NS2, NS3/NS3a and NS4)

The two larger BTV non-structural proteins (NS1 and NS2) are expressed at high levels in both insect and mammalian cells, while the smallest non-structural proteins (NS3 and NS3a) are barely detectable in infected mammalian cells. However NS3 and NS3a are synthesised at high levels in insect cells (Guirakhoo et al., 1995). Recently a fourth highly conserved BTV non-structural protein (NS4) has been identified in both insect and mammalian cells infected with BTV, encoded by an out-of-frame down stream ORF on Seg-9 (Belhouchet et al 2011).

NS1 and NS2 are encoded by Seg-5 and Seg-8 respectively, which are highly conserved between different BTV strains, although they do show significant sequence variations that correlate with the geographic origins of the virus isolate (Seg-5 and Seg-8 topotypes) (Maan et al., 2008). In contrast NS3 and NS3a are encoded by Seg-10, which is the fourth most variable of the BTV genome segments, seperating into different clades that show only a partial correlation with topotype (Shaw et al., 2007; Bonneau et al., 2001; Maan et al 2010) . Seg-10 of African horse sickness virus AHSV

is the second most variable protein after VP2 (Anthony et al., 2007; Van Staden and Huismans 1991).

NS1: is encoded by Seg-5 (Mertens et al 1984; Shaw et al., 2007). It forms into virus specific tubules that are abundant in BTV infected cells (Huismas ans Els 1979). The assembly of tubules may result from the presence of several very strongly hydrophobic regions in the NS1 protein (Roy, 1992a; Schwartz-Cornil et al., 2008). The role of the NS1 tubules has not yet been fully determined, although recent studies suggest that they may play some role in controlling the release of BTV progeny virus particles from infected cells (Prassad et al., 1992; Monaco et al., 2006), or in control of translation in infected cells (Mark Boyce personal communication) . Monaco et al., (2006) developed a diagnostic RT-PCR assay to distinguish the Italian BTV-16 (NS1) gene from that of the homologous vaccine strain. This followed findings where Seg-5 showed the highest differences (17.3%) between these two virus strains in comparison to variations found in segment 2, 6 and 10. However, recent studies have shown that the Italian strain of BTV-16 from 2002, is a reassortant virus, containing genome segments derived from parental strains of the BTV-2 and BTV-16 vaccines (Batten et al., 2008). Consequently, although most of the genome segments of the italian BTV-16 strain belong to an eastern topotype, the NS1 gene belongs to a western topotype, explaining the large sequence differences that were observed.

NS2(ViP): is encoded by Seg-8 (Ross-Smith et al., 2008), which is highly conserved, although it still seperates into distinct eastern and western clades / topotypes (Maan et al., 2008). NS2 is a major constituent of the viral inclusion bodies (VIB) that form in the cytoplasm of BTV infected cells. These structures represent the site of BTV RNA synthesis, core assembly and genome packaging (Schwartz-Cornil et al., 2008; Noad and Roy, 2009; Ross-Smith et al., 2008). NS1 and NS2 are both highly conserved and are antigenically cross-reactive between different strains, serotypes and topotypes of BTV (Jones et al., 1997).

NS3 and NS3a: The NS3 and NS3a proteins are collinear translation products of Seg-10. They are glycoprotein components of the cell membrane with two transmembrane domains and a short extracellular central region (Bonneau et al., 2001). These non-structural, membrane proteins play a role in virus egress from both the mammalian and insect cells (Bonneau et al., 2001; Elia et al., 2008; Hyatt et al., 1993). NS3 act as a

viroporin, facilitating virus release by inducing membrane permeabilisation, allowing BTV particles to leave the host cells (Schwartz-Cornil et al., 2008)

NS4: Although each genome usually encodes a single protein from a single open reading frame (ORF). However, (Belhouchet et al., 2011) recent analyses identified a new overlapping ORF in segment 9 (Seg-9) of both insect-borne and tick-borne orbiviruses, confirming the existence of a new and previously un-described protein identified as NS4 thus this segment encodes two viral proteins (VP6 and NS4). NS4 is highly conserved among several BTV serotypes/strains with a length of approximately 77–79 amino acid. It is early days to determine the role of this protein, however it's association with lipid droplet may play a role in the cell exit of the virus, on the other hand identification of this protein in the nucleus by which BTV can counteract the defences of the host via interference with the innate immune response (Belhouchet et al., 2011; Ratinier et al., 2011)

Table 1-1 BTV genome segments and proteins:

Segment (bp)	Protein	Size(kDa) Copy number	Function (location)
1(3954)	VP1(Pol)	149/10	RNA dependent RNA polymerase
2(2926)	VP2	111/180	Outer layer of the outer capsid, controls virus serotype, cell attachment protein, involved in determination of virulence, readily cleaved by proteases. Most variable protein. Reacts with neutralizing antibodies.
3(2770)	VP3(T2)	103/120	Forms the innermost protein capsid shell sub-core capsid layer, controls overall size and organisation of capsid structure, RNA binding, interacts with minor internal proteins.
4(2011)	VP4 (Cap)	76/20	Dimers, capping enzyme (guanylyltransferase), transmethylase 1 and 2.
5(1769)	NS1 (TuP)	64/0	Forms tubules of unknown function in the cell cytoplasm. These are a characteristic of orbivirus replication
6(1638)	VP5	59/360	Inner layer of the outer capsid, glycosylated, helps determine virus serotype, variable protein.
7(1156)	VP7 (T13)	38/780	Trimer, forms outer core surface, T=13 symmetry, in some genera (AHSV) it can form flat hexagonal crystals, involved in cell entry and core particle infectivity in adults and cells of vector insects, reacts with core neutralising antibodies, Immuno dominant serogroup (virus species) specific antigen.
8(1124)	NS2 (ViP)	41/0	Important viral inclusion body matrix protein , ssRNA binding, phosphorylated. Can be associated with outer capsid.
9(1046)	VP6 (Hel) VP6a	35/60	ssRNA and ds RNA binding, Helicase, NTPase.
	NS4	12/0	Nonstructural, role not yet determined, believed to counteract the antiviral response of the host to BTV-8.
10(822)	NS3 NS3a	25/0 24/0	Glycoproteins, membrane proteins, involved in cell exit, in some genera (AHSV) these are variable proteins and are involved in determination of virulence.

Table 1.1: List of the dsRNA segments of BTV-10 with their respective sizes (bp) and their encoded proteins for which the name, calculated size (kDa) and function and/or location are indicated [adapted from http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ and Mertens et al., 2000)]

1.4 : Bluetongue disease

1.4.1 : Clinical signs in sheep

Bluetongue is primarily a disease of sheep and all breeds are thought to be susceptible, although to variable degrees (Erasmus, 1975). Marino sheep are considered to be especially susceptible (MacLachlan, 1994), although animals belonging to this breed, reared in South Africa for several generations, have become much more resistant. Sheep breeds from non-endenic areas (such as northern Europe) are usually naïve, and are therefore significantly more susceptible than those from regions where the virus is continually circulating. However, there are also variations in the response of individual animals to BTV infection, ranging from innapparent to fatal (Mertens and Mellor, 2003; Bowne, 1971; OIE, 2004). These variations can depend on age, health, immunological status (due to vaccination or previous infection), immunodeficiency due to concurrent infections, and environmental factors such as sunlight, and the availability of either food and water (Haig, 1959; Schwartz-Cornil et al., 2008). There can also be significant variations in the incubation period before development of clinical signs following artificial infection of sheep (2-15 days), with an average of 4-6 days, while the incubation period following natural infection is thought to be slightly longer (approximately 7 days)(Erasmus, 1975).

The first signs of BT are fever, followed by an increase in respiratory rate with hyperaemia, swelling of the face, lips and tongue, which occasionally leads to cyanosis of the tongue, after which the disease is named (Maan et al., 2008). There can be petechial haemorrages of the buccal and nasal mucosa with excessive frothing salivation, nasal discharge, crusting and ulceration of the muzzle, with oedema of the lips and inter mandibular space (Mahrt et al., 1986; Thomas et al., 1947; Erasmus, 1979; Enserink, 2008). BTV infected sheep may also develop severe coronitis and muscular weakness, developing a stiff gait, and may be reluctant to move. They can also become emaciated, with wool break and the entire fleece can fall off (Thomas et al., 1947, Haig, 1959). Severely affected sheep sometimes vomit rumen contents through the nose and mouth. When a sheep is observed to have rumen contents escaping from it's nostrils and accumulating around it's lips, death of the animal from foreign body pneumonia is predicted within 48 hours (Bowne, 1971).

1.4.2 : BT in cattle

BTV infection of cattle is usually asymptomatic but BTV has a haemagglutination activity, and consequently infection can cause a long lived viraemia (particularly in cattle), due to virus particles adherring to circulating erythrocytes.

Cattle serve as a source of the virus, infecting vector insects and allowing the virus to be transmitted to other ruminants (MacLachlan, 1994; Veronesi et al., 2005; Stott et al., 1990; Barratt-Boyes et al., 1992). During the northern European outbreak 2006-2008 caused by BTV-8, a low percentage of infected cattle (~10%) were clinically affected, showing typical clinical signs, including reduced milk production, fever, congested mucous membrane, purulent nasal discharge, profuse salivation, lameness and ulceration of the teats (Dercksen and Lewis, 2007; Shaw et al.,2007), with case fatality rates reaching approximately1.0%. This may be partially due to incursion of the virus into a serological naïve cattle population, but also suggests the presence of a particularly virulent strain of the virus (Darpel et al., 2007). In addition the virus was transmitted vertically in cattle, causing teratogenic effects (dummy calves) (Vercauteren et al., 2008; Darpel et al., 2009; Williamson et al., 2010)

In non domesticated ruminants the disease can vary from acute haemorrhagic disease with high mortality rate, in white tailed deer (Odocoilus virginianus), to an inapparent as seen in the North American elk (Cervus Canadensis) (OIE, 2004).

1.5 : Bluetongue disease significance

Bluetongue is classified by the Office International Des Epizooties (OIE) as a notifiable disease (former List A) (Di Emidio et al., 2004; Monaco et al., 2006; Maan et al., 2007a; Darpel et al., 2007). These are communicable disease that have the potential for very serious socioeconomic or public health consequences, which are of major importance to the international trade of livestock and livestock products. Affected countries are therefore required to inform the OIE of any new outbreaks (Boone et al., 2007; Darpel et al., 2007; Monaco et al., 2006).

Bluetongue is thought to have originated in Africa and was recorded in 1876 following the introduction of highly susceptible Marino sheep into the cape colony (Erasmus, 1975; Howell, 1963; Haig, 1959). Bluetongue occurs between latitude 40° S and 53° N in the Americas, Africa, Asia and Australia (Anthony et al., 2007; Mertens et al., 2007). Prior to 1998 BTV had only caused sporadic, periodic and relatively short lived

epizootics within southern Europe involving a single serotype on each occasion (Anthony et al., 2007; Purse et al., 2005; Elia et al., 2008). However, since 1998, there have been at least 14 separate introductions of BTV into Europe, involving at least eleven viral strains belonging to nine different serotypes (type1, 2, 4, 6, 8, 9, 11, 14 and 16) (Mertens et al., 2007; Hamers et al., 2009), and most recently the emergence of BTV-14 in Russia, ascribed to the illegal use of modified live vaccines (Eschbaumer et al., 2009 , javascript:pre('1020349', '', 'Bluetongue')

Recent outbreaks of BTV in Europe started with a report during October 1998, of BTV-9 in sheep on several Greek islands. This was followed by an outbreak in June 1999 in Bulgaria (BTV-9). By July BTV-4 was detected in European Turkey, BTV-4 and 16 were reported in Greece and serotype 2 was detected in Tunisia and Algeria, then spread to Sardinia ,Calabria, Sicily (Hammoumi et al., 2003). The range of BTV gradually extended further northwards into Mediterranean and central Europe (as far as 44°N) (Anthony et al., 2007), causing one of the largest epizootics of bluetongue ever recorded, with the death of >1.8 million animals (mainly sheep) (Purse et al., 2005; Maan et al., 2007a; Shaw et al.,2007; Darpel et al., 2007).

The spread of BTV into Mediterranean Europe is believed to be a consequence of climate change and global warming, which appear to have altered the distribution of the major vector species *Culicoides imicola* in southern Europe (Anthony et al., 2007, Purse et al., 2005; Maan et al., 2007a; Shaw et al., 2007; Wilson et al., 2007; Mertens et al., 2007). However, *C. imicola* is absent from northern Europe and BTV transmission in this region must therefore involve alternative and novel vector species (Dungu et al., 2004). *C. pulicaris* and / or *C. obsoletus* are abundant across the whole of northern Europe including the UK and are thought to have been involved in the outbreak caused by BTV-8 during 2006 to 2010 (Takamatsu et al., 2003; Shaw et al., 2007).

In August 2006 the first outbreak ever recorded in northern Europe started in Belgium, and the Netherlands, spreading to Luxemburg, Germany and north east France. This outbreak was caused by BTV-8 (Maan et al., 2008; Wilson et al., 2007; Dercksen and Lewis, 2007; Mertens et al., 2007). BTV-8 was also confirmed on September 15th 2007 in a farm in Suffolk (UK) (Gloster et al., 2008). This BTV-8 strain belongs to a western topotype and is most closely related to BTV-8 strains from (Nigeria). (Maan et al 2008), although it is not clear how it arrived in northern Europe. It is also unclear

how the virus survived the vector free period (overwintered) to re-emege in 2007 (Fabiana et al., 2009). However, (persistent BTV infection of ovine γδT cells has been suggested by (Takamatsu et al., 2003). It has also been suggested that vertical transmission of BTV in the insect vector could also be involved (White et al., 2005). Vertical transmission was observed as a frequent event in cattle infected with BTV-8 (Santman-Berends et al., 2007), and horizontal (oral) transmission of BTV-8 was observed in Ireland (Szmaragd et al., 2007). Backx et al., (2009) studied the transplacental transmission (vertical) of wild-type BTV-8 in late gestation and infection of the neonate by infected colostrums (horizontal). The findings supported the possibility of alternative transmission routes for BTV-8 that may explain its overwintering between vector seasons.

Another possibility for (overwintering) is the survival of small numbers of adult midges in cowsheds during the winter, which has been recorded in Belgium, although there was no evidence that these midges were infected with bluetongue (Menzies et al., 2008). The movement of cattle within Europe has also resulted in the importation of BTV-8 infected animals into new areas, resulting in outbreaks in Spain and Italy in 2007-2008 (Maan et al., 2008).

It is estimated that during 2007 Belgium alone lost 12-15% of its national sheep flock killed by BTV, with total costs to the European cattle and sheep industries exceeding £100 million. There were also losses caused by reduced productivity and movement bans in affected livestock (Gloster et al., 2008; Savini et al., 2007). BT is economically important not only because of direct losses caused by the disease, but also as a result of indirect losses, due to bans on livestock movement (Pathak et al., 2008). Restrictions were imposed of the international trade in ruminant semen and embryos, due to the risks of BTV transmission through bovine germplasm, during embryo transfer procedures and the possibility that infected bulls could excrete virus in their semen. There were also loses caused by congenital and teratogenic BTV infections in cattle, resulting in hydranencephaly with moderate to severe CNS disorders ('dummy calf syndrome'), growth retardation, emaciation incompatible with life and losses caused by abortion (Fabiana et al., 2009). In addition to these problems, the imposed requirements for surveillance, certification and quarantine (60 days for BTV) prior to movement of animals, represent further costs to the livestock industries (Lunt et al., 2006).

In an attempt to reduce these direct and indirect losses and to allow safe movement of animals from endemic areas, vaccination strategies were developed. These have varied in different countries according to their individual policies, the geographical distribution of the BTV serotypes involoved and the availability of appropriate vaccines (Prasad et al., 1992; Savini et al., 2007).

1.6 : Bluetongue disease control

Bluetongue disease control strategies have varied in different countries however, the most frequently adopted control meassures include:

- clinical and serological surveillance
- animal movement restrictions
- slaughter of infected and seropositive animals,
- insecticide treatment of infected and "at risk" premises
- mass vaccination (Mellor et al., 2008).

Because, bluetongue is a non-contagious viral disease that is spread by infected biting-midges of the genus *Culicoides*, control measures that are based on culling of infected livestock are not considered to be effective as a control messure when used alone. Although control or restriction of access by vector insects could be effective, these insects are usually abundant and ubiquitous during at least part of the year.

Methods of controlling Culicoides spp. (Diptera: Ceratopogonidae) Carpenter et al., 2008) have included:

- application of insecticides to habitats where larvae develop.
- environmental interventions to remove larval breeding sites
- controlling adult midges by treating either resting sites, such as animal housing, or host animals with insecticides
- housing livestock in screened buildings
- using repellents or host kairomones to lure and kill adult midges

However, because the virus replicates in both the insect vector and in a range of ruminants, including cattle and goats (often with prolonged viraemia and less-severe disease symptoms), once an outbreak has become established control is difficult using these measures alone. Also, the effect of implementing effective husbandry and

insecticidal treatments to control midges must be weighed against the costs to livestock producers and government agencies, and the potential environmental or human health risks incurred. Realistic estimates are therefore required of the socio-economic impact of BTV on beef, dairy and sheep producers in northern Europe, to estimate the costs and benefits of implementing control techniques (Carpenter et al., 2008; Roy et al., 2009).

Another factor is that different species of *Culicoides* prefer different feeding sites. *Culicoides imicola* has been reported to feed on the animal's back while *Culicoides sonorensis* feeds on the belly, and other species may feed on the lower legs. It is therefore important to establish whether commercial pour-on insecticides, which are most commonly applied along the dorsum of ruminant livestock, will give sufficient protection from biting midgse at the feeding sites (Papadopoulos et al., 2009). The control of bluetongue, by control of adult *Culicoides* may therefore be impractical.

In an attempt to reduce direct and indirect losses due to bluetongue, vaccination of susceptible species is regarded as the method of choice and permits a safe trade in live ruminants, under certain conditions (Gethmann et al., 2009).

1.7 : Vaccines

1.7.1 : Historical Perspective

The discipline of immunology grew out of the observation that individuals who had recovered from certain infectious diseases were thereafter protected against reinfection with the same, and sometimes against related diseases. The Latin term *immunis,* (meaning "exempt") is the source of the English word immunity, meaning the state of protection from infectious disease.

Perhaps the earliest written reference to the phenomenon of immunity can be traced back to Thucydides, the great historian of the Peloponnesian War. In describing a plague in Athens, he wrote in 430 BC that only those who had recovered from the plague could nurse the sick because they would not contract the disease a second time (Kurby, 2000). The first recorded attempts to induce immunity deliberately, were performed by the Chinese and Turks in the fifteenth century. Various reports suggest that the dried crusts derived from smallpox pustules were either inhaled into the nostrils or inserted into small cuts in the skin (a technique called *variolation*). In 1718, Lady Mary Wortley Montagu, performed the technique on her own children. The

method was significantly improved by the English physician Edward Jenner, in 1798. Intrigued by the fact that milkmaids who had contracted the mild disease cowpox were subsequently immune to the disfiguring and often fatal disease smallpox, Jenner reasoned that introducing fluid from a cowpox pustule into people (i.e., inoculating them) might protect them from smallpox, and indeed this proved to be effective. The term vaccination is derived from these early experiments (from vaccusa meaning cow).

The next major advance in immunology was the induction of immunity to Cholera by Louis Pasteur. Pasteur hypothesized and proved that aging the bacteria causing Cholera, reduced the virulence of the pathogen and that such an attenuated strain could be administered to protect against the disease. Pasteur extended these findings to other diseases, demonstrating that it was possible to attenuate, or weaken a pathogen and administer the attenuated strain as a vaccine.

1.7.2 : Host immune responses and vaccination

Recent advances in immunology and molecular biology have led to more effective new vaccines and to promising strategies for delivering vaccine antigens and developing new vaccine candidates (Kurby, 2000). B and T lymphocytes recognize discrete sites on an antigen, which are known as 'antigenic determinants' or 'epitopes'. Although B cells can recognise an epitope alone, T cells can recognize an epitope only when associated with a MHC molecule on the surface of a self-cell (either an antigen-presenting cell or an altered self-cell). Each branch of the immune system is therefore uniquely suited to recognize antigens in a different milieu. The humoral branch (B cells) recognizes an enormous variety of epitopes: those displayed on the surfaces of bacteria or viral particles, as well as those displayed on soluble proteins, glycoproteins, polysaccharides, or lipopolysaccharides that have been released from invading pathogens. However, the cell-mediated branch of the immune system (T cells), recognizes protein epitopes displayed together with MHC molecules on self-cells, including altered self-cells, such as virus-infected cells and cancerous cells. Thus, in addition to innate immune recognition, four related but distinct cell-membrane molecules are responsible for antigen recognition by the immune system. Including: Membrane-bound antibodies on the B cells surface immunoglobulin [SIg] or BCR; T-cell receptors (TCR); Class I MHC molecules; Class II MHC molecules

Each of these molecules plays a unique role in antigen recognition, ensuring that the immune system can recognise and respond to the different types of antigen that it

encounters. Knowledge concerning differences in the epitopes recognized by T cells and B cells has helped immunologists to design vaccine candidates to maximize activation of both arms of the immune system. As differences in antigen-processing pathways became evident, scientists began to design vaccines and to use adjuvants that maximize antigen presentation with class I or class II MHC molecules (Kurby, 2000).

Innate immune responses play a role in immediate defence against pathogens. These responses and pathogen recognition are genetic rather than acquired. Recognition is mediated by several families of pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) and C-type lectin receptors to identify particular pathogen associated molecular patterns (PAMPs). Recognition of PAMPs (or so called danger signals) initiate secretion of cytokines (such as interferon [IFN]), induction of inflammation, immune cell recruitment, antigen uptake and presentation of antigen by dendritic cells (DC), which are essential for initiating acquired immune responses. The innate immune response to viral infection is primarily through the induction of type I IFNs (IFN-α and IFN-β) and the activation of NK cells. Double stranded RNA (dsRNA) produced during the viral life cycle can induce the expression of IFN-α and IFN-β by the infected cell through intracellular TLR 3, 7 and 8. Macrophages, monocytes, and fibroblasts also are capable of synthesizing these cytokines, but the mechanisms that induce the production of type I IFN in these cells are not completely understood.

IFN-α and IFN-β can induce an antiviral response or resistance to viral replication by binding to the IFN -α/β receptor which in turn induces the synthesis of both 2-5(A) synthetase and protein kinase (PKR), which activate a ribonuclease (RNAse L) that degrades viral mRNA. PKR inactivates the translation initiation factor. Both pathways thus result in the inhibition of protein synthesis and thereby effectively block viral replication in infected cells. The type of vaccine and /or adjuvant which is used, and their capability to induce the correct type of innate responses, also influences their ability to generate the desired adaptive immune response. The choice of an appropriate antigen adjuvant combination therefore becomes an important factor in vaccine design.

The main purposes of BTV vaccination strategies (Savini et al., 2007).are:

1) Prevent initial infection of the mammalian host.
2) Prevent or reduce replication of the virus within the mammalian host.
3) Protect the mammalian host from clinical disease.

4) prevent onward transmission of the virus and limit the regional extension of BTV infection.
5) reduce persistence of the virus in the vaccinated host.
6) allow regional eradication of the disease based on reduction of virus circulation
7) authorise the safe movement of susceptible animals between affected and free zones.

1.7.3 : Vaccine classifications

There are four main types of existing or potential BTV vaccines, these include: attenuated live virus vaccines, inactivated vaccines, subunit vaccines and DNA vaccines.

a) Live attenuated vaccines

Attenuation of BTV virulence can be achieved by multiple serial passage of the virus in embryonated chicken eggs, or mammalian tissue culture cells (e.g. BHK cells). This process selects viruses with a reduced capacity to grow *in vivo* and consequently to cause disease in vaccinated animals (Prasad et al., 1992; Monaco et al., 2006; Savini et al., 2007). These vaccines are cheap, and easy to produce while generating strong, long-lived protective immunity against challenge with virulent homologous virus , after a single inoculation, (Breard et al., 2007; Schwartz-Cornil et al., 2008; Di Emidio et al., 2004; Murray and Eaton, 1996).

Twenty one of the twenty six known serotypes of BTV have been detected in South Africa. Strains of several of these types have a high pathogenic index and high epidemic potential, including serotype 1,2,3,4,6 and 10. Vaccination is recognised as an effective means of controlling the disease (Dungu et al., 2004). The attenuated vaccines produced by Onderstepoort Biological Products (South Africa) have long been used to control Bluetongue in sheep in Southern Africa and more recently in Corsica, the Balearic Islands and Italy (Schwartz-Cornil et al., 2008; Veronesi et al., 2005). These vaccines are produced in cell culture and supplied as a freeze-dried polyvalent preparation, which is administered subcutaneously. The vaccine comprises three bottles (A, B and C), which are administered separately at three weeks intervals (Dungu et al., 2004; Alpar et al 2008).

Bottle A: contains BTV serotype 1, 4, 6, 12 and 14

Bottle B: contains BTV serotype 3, 8, 9, 10 and 11

Bottle C: contains BTV serotype 2, 5, 7, 13 and 19.

Jeggo et al., (1984) discovered that the simultaneous inoculation of sheep with three different BTV types (type 3, 4 and 6) resulted in the replication of only two of these types (3 and 4) and formation of neutralising antibodies to only these two types. This could be because the two types induced high levels of cross-reactive cytotoxic T lymphocytes which curtailed replication of the third type (BTV type 6). Similar results were obtained in a murine model (Jeggo and Wardley 1982c). Co-Inoculation of different BTV strains / serotypes can result in reassortment of genome segments and the emergence of novel strains. The use of multivalent vaccines in areas where a number of serotypes co-exist, should therefore be reappraised.

Live vaccines often generate a stronger and more long-lasting protective immunity response than inactivated vaccines. This may be due to a larger effective antigenic mass, generated by the *in vivo* replication of the virus, and a greater efficiency of antigen presentation in association with the host's MHC major histocompatibility complex molecules on the antigen presenting cells. This association is essential for effective induction of adaptive immunity (Pearson and Roy 1993). However there are some serious risks associated with the use of live vaccination.

i) Risks associated with attenuated BTV vaccines

1) Veronesi et al., (2005) tested a number of commercially available live vaccines, (produced by Onderstepoort Biological Products, in South Africa), .in European Dorset Poll sheep. These animals developed severe clinical signs of BT following vaccination.

2) Live vaccine viruses caused viraemia in vaccinated animals, which can be sufficient to infect blood-feeding vector *Culicoides* (Veronesi et al., 2005; Veronesi et al., 2010). However, in some studies the level of vaccine-viraemia was low and although the virus was detected by RT-PCR. it was not possible to reisolate it after vaccination (Breard et al., 2007, Hammoumi et al., 2003),

3) Live attenuated vaccine viruses can replicate in and can be transmitted by vector *Culicoides* (Murray and Eaton 1996; Veronesi et al., 2005; Ferrari et al., 2005). Venter et al., (2007) demonstrated that *C. imicola* and *C. bolitinos* can both be infected by and support the replication of at least 12 out of 16 attenuated BTV strains used in the

current production of the commercial polyvalent vaccines in South Africa. Similar results were obtained by when two vaccine viruses (BTV-2 and 9) were used in oral infection studies, in two species of *Culicoides*, (*C. sonorensis*, and *C. nubeculosus),* reaching titres that indicate transmission would occur. (Veronesi et al., 2005),

4) It is considered possible that vaccine viruses could revert to virulence on passage through vector *Culicoides* (Venter et al., 2007; Schwartz-Cornil et al., 2008, Veronesi et al., 2005; Ferrari et al., 2005; Savini et al., 2007).

5) Live vaccine viruses can echange genome segments (reassort) with wild–type or other vaccine viruses in infected and/or vaccinated animals or vector *Culicoides*, giving rise to novel BTV strains, that could potentially have modified virulence characteristics (Veronesi et al., 2005; Ferrari et al., 2005; Batten et al 2008).

6) Modified live BTV vaccine strains have been shown to cross the ruminant placenta, with the timing of foetal infection determining outcomes, ranging from cerebral malformations, to foetal death, abortion and resorption (MacLachlan et al., 2000; Breard et al., 2007; Di Emidio et al., 2004) .

7) Modified live BTV vaccines could also have a significant affect on the quality of ram semen and milk production (Breard et al., 2007; Schwartz-Cornil et al., 2008).

8) Although Live BTV vaccines may protect the sheep against disease, they do this by causing infection. Consequently the attenuated BTV strains cannot be used as DIVA vaccines (Breard et al., 2007; Schwartz-Cornil et al., 2008).

Several authors have reported oligonucleotide primers that can be used to amplify and distinguish specific genome segments from either vaccine or wild type bluetongue viruses, but with no clear knowledge of the genetic basis for attenuation, these assays simply detect differences between different virus strains, rather than positively idenitifying strains that are attenuated (Aguero et al., 2002; Monaco et al., 2006; Breard et al., 2003; Mertens et al 2007).

b) Inactivated virus vaccines

Inactivation of BTV can be accomplished by the use of binary ethylenimine for 48 hours at 37°C (Odeon et al.,1999). However, this can cause some alteration of the antigenic characteristics of VP2 (Stott et al.,1985; Odeon et al.,1999). Di Emidio et al., (2004) used B-propriolactone at a concentration of 0.2% (v/v) for inactivation of

BTV-2, with very good results. To confirm total inactivation, the treated BTV was inoculated into monolayers of Vero cells and embryonated chicken eggs (Odeon et al., 1999). The first trials to develop inactivated vaccines against BTV prepared from whole virus, goes back to 1975 (Alpar et al., 2008).

Providing they are properly produced, killed BTV vaccines can generate a safe and protective immunity, and cannot be transmitted to other animals in the field (Murray and Eaton 1996; Schwartz-Cornil et al., 2008; Di Emidio et.al 2004). However, these vaccines are not 100% effective and typically require repeated immunisation in cattle (at least twice), possibly reflecting a loss of immunogenicity associated with inactivation (Stott et al., 1985; Murray and Eaton 1996; Di Emidio et al., 2004; Boone et al., 2007). It has been suggested that 80% seroconversion would be sufficient to halt a BTV outbreak, suggesting that even if these inactivated vaccines are less than 100% effective they would still provide a valuable control measure (as seen in the UK during 2008).

Inactivated BTV vaccines can induce neutralising antibodies, but unlike the live attenuated viruses they need adjuvants (Elia et al., 2008; Savini et al., 2007). They are also considered to be the best (safest) vaccines currently available for use in Europe. This is based on both safety and efficacy levels (Schwartz-Cornil et al., 2008), with few if any side effects (such as the risk of abortion, decreased milk production reassortment or reversion to virulence) (Di Emidio et al., 2004; Alpar et al., 2008). Consequently the European Food and Safety Authority recommended the use of these vaccines, and the United Kingdom, France, Germany and the Netherlands used inactivated BTV-8 vaccines to protect sheep and cattle, and halt the spread of the BTV-8 outbreak during 2008/2009 (Enserink, 2008).

Assays to differentiate infected from vaccinated animals (DIVA) may be possible for purified and inactivated BTV vaccines (Savini et al., 2007). However, the inactivated vaccines that were used used in Europe, contain only partially purified, or unpurified BTV proteins derived from infected cell cultures. They therefore contain all of the structural and non-structural proteins, suggesting that DIVA assay development may be difficult or impossible. Inactivated vaccines are currently available for only a few of the BTV serotypes (Schwartz-Cornil et al., 2008).

After its introduction in 2006, BTV-8 caused a severe epizootic in north-west and Central Europe, in areas where BTV had never previously been reported and which were not usually considered to be at risk of BTV introduction. As a result, a compulsory vaccination program was carried out in Germany in 2008 against BTV-8, using inactivated monovalent vaccines (Eschbaumer et al., 2009). Gethmann et al., (2009) tested the safety of three BTV-8 inactivated vaccines produced by three different manufacturers (BLUEVACR 8, CZ Veterinaria, Spain; BTVPURR AlSap 8, Merial, France; and ZulvacR 8 Ovis or Bovis, respectively, Fort Dodge, The Netherlands) in a trial carried out on sheep and cattle. All vaccines in this study provided high levels of safety. Vaccination of cattle with a bivalent BTV-2 and BTV-4 inactivated vaccine, also resulted in a complete prevention of detectable viraemia in all calves when challenged with high doses of BTV-2 or BTV-4 (Savini et al., 2009)

Hamers et al., (2009) studied the duration of protective immunity in sheep vaccinated with an inactivated bluetongue serotype-2 vaccine, and concluded that a single dose of the BTV-2 vaccine was sufficient to provide protection for at least one year. However, one of the drawbacks with killed whole cell vaccines is the dangers posed by the growth of large cultures of infectious virus before inactivation. It is also important that the virus be completely inactivated in every vaccine batch, as otherwise vaccination could lead to some of the problems discussed above for attenuated vaccines (Roy et al., 2009)

A number of potential alternatives have been investigated to address the unmet requirements for an improved bluetongue vaccine, i.e. low cost, ability to distinguish between vaccinated and infected animals, broad protective immunity, full protection of individual vacinees, prevention of transmission, protection against multiple serotypes, long lasting immunity and preferably, a single dose.

c) Subunit vaccines

The basic principle for a subunit vaccine is that the gene encoding the subunit vaccine is isolated and transferred to a second, normally non-pathogenic organism or virus. The recombinant subunit vaccine is then produced by the heterologous host, and can be designed to be delivered, either as a purified immunogen, or by using the production host as a live vector (Liljeqvist and Ståhl 1999). For a subunit vaccine to be a realistic vaccine candidate, it needs to be produced in a safe and efficient

expression system that is amenable to large scale production and antigen purification(Noad and Roy, 2003) .

The advantages of recombinant subunit vaccines are numerous. The pathogen can be entirely excluded from the production of the vaccine, eliminating risks associated with production, including: incomplete inactivation of whole-cell vaccines, contamination with toxic compounds used for inactivation, or reversion to virulent genotypes / reassortment / transmission of live vaccines. Although subunit vaccine candidates may not bet optimally immunogenic, this can often be enhanced by improving the expression system or delivery vector (Liljeqvist and Ståhl 1999).

BTV-VP2: (the most variable of the viral proteins) is responsible for virus neutralisation and serotype determination. A range of different approaches have therefore been used to assess the protective efficacy in animals, of either VP2 by itself, or in combination with other viral proteins (Roy et al., 2009). A combination of VP5 with VP2, not only protected all of the vaccinated sheep, but elicited a higher neutralising antibody response, compared to VP2 alone, whether expressed by recombinant baculovirus, or in canarypox vaccine vector (Roy et al., 1990). The immunological similarity of baculovirus expressed virus like particles (VLP – composed of VP2, VP3, VP5 and VP7) to native BTV particles suggests that they are able to induce both B-cell mediated responses as well as a cytotoxic T lymphocyte (CTL) response, although these are only against the virus structural proteins (Noad and Roy, 2003).

Genetic engineering techniques can be used to develop subunit vaccines without the need to grow the pathogenic organism itself, as a source of antigen components (Roy et al., 1990). Huismans et al., (1987) developed a method to purify the neutralisation specific antigen of bluetongue virus (VP2) in large amounts, which was subsequently injected into sheep. An initial dose of 50 μg of VP2 was sufficient to induce VP2-specific antibodies that precipitated the protein, neutralized the virus and inhibited hemagglutination. The sheep that had received the purified VP2 were fully protected against challenge with a virulent strain of the same BTV serotype. These observations suggested that VP2 could be used as an effective subunit vaccine and the synthesis of VP2 by recombinant-DNA technology was explored (Huismans et al., 1985). In a trial carried out by (Roy et al., 1990) a dose of 50μg of VP2 expressed by recombinant

baculovirus, provided protection for some sheep. However, a dose exceeding 50μg/sheep provided protection for all of the sheep.

VP7(T13): The immune protection generated in respose to the cross-reactive and BTVserogroup-specific antigen VP7(T13) was tested by Wade- Evans et al., (1996). Six out of eight sheep vaccinated with BTV-1 VP7 were protected when challenged with BTV-3, providing evidence of cross-protection. Since NS1 is also conserved between BTV serotypes and is recognised by cross-reactive CTL, it may also represent a useful subunit vaccine component, that could potentially stimulate cross-serotype protective responses (Andrew et al., 1995).

VLPs: These are self-assembled virus-like-particles composed of one or several viral proteins or peptides, expressed *in vitro* through recombinant technologies. The self-assembly property of these proteins for different viruses results in the formation of particles ranging in size from about 20–100 nm, which, have in some cases provided an effective alternative to live virus vaccine (Noad and Roy, 2003; Scheerlinck and Greenwood, 2008). Recombinant expressed BTV virus-like-particles contain the four major structural proteins of VP2, VP3, VP5 and VP7 (Belyaev and Roy, 1993; Roy et al., 1997). While co-expressed VP3 and VP7 of BTV can form core – like particles (Roy et al.,1997).

One of the earliest vaccines based on this concept was produced using the hepatitis B surface antigen (HBs Ag) expressed in yeast which generated particles that were similar to the 22nm particle secreted by infected human cells. This vaccine was first licenced in 1986 (Alpar et al., 2008; Noad and Roy, 2003; Liljeqvist and Ståhl 1999). The second commercially available VLP vaccine is composed of the major capsid protein of the human papilloma virus (HPV) L1 (Scheerlinck and Greenwood, 2008) .

Like many other subunit vaccines, VLP based vaccines are based on an understanding of the method of entry and infection of cells by the virus. In BTV, VP2 (the major outer capsid protein) mediates virus attachment to the cell, while VP5 (the second major outercapsid protein) is critical for the pH dependant release of the viral cores from the endosome compartments (Roy, 2003). After entry into the cells, the virus is uncoated (VP2 and VP5 are removed) to release the transcriptionally active core particle (Hassan et al., 2001). BTV-VLP mimic the overall structure of the virus particle, while completely lacking the BTV RNA genome. Their conformation is therefore close to that of the authentic viral capsid, but without the risk (as seen with

the live attenuated vaccines) of virus replication, re-assortment and transmission in the field (Noad and Roy, 2003; Pearson Roy, 1993, Murray and Eaton, 1996)). The immunological similarity of VLP to native BTV particles, suggests that they are able to induce both B-cell mediated and cytotoxic T lymphocyte (CTL) responses, although these will only be against the virus structural proteins (Noad and Roy, 2003).

The combination of 20μg of VP5 with 50μg of baculovirus expressed VP2, not only protected all of the vaccinated sheep, but elicited a higher neutralising antibody response compared to the respose following vaccination using VP2 alone (Roy et al., 1990). However, as little as 10μg of VLP / sheep gave long lasting protection (14 months) when challenged with homologous serotype (Roy et al.,1997; Noad and Roy, 2003). Since the core proteins of CLP are conserved across the twenty six BTV serotypes, CLP have the potential to generate a cross-reactive vaccine (Alpar et al., 2008). It is also important to note that the formation of CLP from two major BTV proteins and VLP from four major BTV proteins, does not require either dsRNA or non-structural proteins (Aguero et al., 2002; Alpar et al., 2008).

d) DNA vaccines

DNA immunisation represents a recent novel approach to vaccination and immunotherapeutic development. Injection of plasmid DNA encoding a foreign gene of interest can result in the subsequent expression of the foreign gene products and the induction of an immune response within a host. This is relevant to prophylactic and therapeutic vaccination strategies when the foreign gene represents a protective epitope from a pathogen (Watts and Kennedy, 1999). DNA vaccine approaches have been widely applied to veterinary infectious diseases including f*eline* immunodeficiency virus (FIV) feline leukaemia virus (FeLV) canine parvovirus, rabies virus, influenza virus and Newcastle disease virus in poultry, Aujeszky's disease, porcine respiratory and reproductive syndrome virus, foot-and-mouth disease virus and influenza A virus in pigs, bovine herpes virus type 1 (BHV-1), bovine respiratory syncytial virus (BRSV) and bovine viral diarrhoea (Dunham, 2002). Currently at least 3 veterinary DNA vaccines are licensed. These are for West Nile virus vaccine in horses (Fort Dodge) (Liu et al., 2006), haematopoietic necrosis virus in salmon (Novartis) (Garver et al., 2005), melanoma in dogs (Merial) (Bergman et al., 2006).

DNA vaccination requires that the plasmid DNA enters a cell, is transcribed and translated, and the foreign gene product needs to be presented as an antigen in tissues

accessible to the immune system. DNA vaccines can be based on bacterial plasmids that have been engineered to express a disease-specific antigen. For successful transfection of the DNA and expression of the antigen, the plasmid should include an efficient promoter to drive transcription of the encoded antigen, one of the most efficient being the human cytomegalovirus (CMV) immediate/early promoter (Dunham, S. P. 2002). They also contain a transcription 'terminator' that is effective in mammalian cells to ensure that the correct mRNA is generated, as well as a selectable marker to facilitate production of the plasmids in transformed bacterial cells . In addition the number and the motif of unmethylated cytidine-phosphate-guanosine (CPG) within plasmid backbone used play a prominent role to enhance immune responses (Gurunathan et al., 2000)

The site of inoculation plays an important role in the induction of protective immune responses to DNA vaccination (Montgomery et al., 1997). As a significantly higher level of protection can be obtained when the DNA is delivered to the skin by methods that enhance DNA uptake and increase transfection efficiency. For example, DNA can be delivered using a gene gun that bombards the skin with gold particles containing adsorbed plasmid DNA. The gold particles directly penetrate the skin due to the force of delivery, thereby increasing the rate of transfection without having to rely on the uptake of DNA by the host cell itself (Watts and Kennedy 1999).These DNA vaccines can also be delivered to the recipient by intramuscular (IM) injection. The DNA is then taken up by host cells and transcribed to mRNA, from which the vaccine proteins are then translated. The antigen is produced within the cells of the vaccinated individual and therefore uses the host's transcriptional machinery (Dunham, 2002; Anderson et al., 2007). The dose of DNA vaccines is generally dependant on the route of adminstration. A dose of 10–100 μg for mice and aproximately 0.5-2.5 mg for larger animals and humans, is required for IM inoculation. In contrast a gene-gun a dose of 0.1-1μg is sufficient in mice to give the same vaccination efficiency. This is probably because plasmids delivered by gene gun are coated onto gold beads, which minimises their degradation, and they are delivered directly into the target cells, which include somatic cells (e.g. myocytes and keratinocytes) and antigen-presenting cells (APCs) themselves (Dunham S.P. 2002)

The mode of action of plasmid DNA vaccines is two-fold. Firstly, the antigen encoded by the plasmid can be produced in host cells, either in antigen presenting cells (APCs)

leading to direct priming of immune responses, or in non-presenting cells from where the antigen can be transferred to APCs leading to cross-priming. Therefore could induce both CD4 (MHC class II presentation) and CD8 (MHC class I presentation) T cell responses. Secondly, because DNA plasmids are derived from bacteria, they contain unmethylated cytosine phosphate-guanosine (CpG) motif. These CpG motifs signal through TLR9 and should therefore stimulate the innate immune system. However, the dose of CpG provided by DNA vaccination may not be sufficient to stimulate TLR9 (Anderson et al., 2007; Liu et al., 2006). The CpG motif has immunostimulatory functions, including: activation of B-lymphocytes to promote antibody secreation; activation of macrophages and dendritic cells to enhance antigen presentation and activation of the cellular responses (Dunham, S. P. 2002).

DNA vaccines have several advantages over more conventional vaccine types, including simplicity of manufacture, biological stability, cost effectiveness, safety, ease of transport in lyophilized form and the ability to act in the presence of maternal immunity and a potential to provide a DIVA strategy, allowing vaccine-induced immunity to be differentiated from post infection immunity (Barros et al., 2009). Different genes could also be combined, for development of multivalent vaccines. DNA vaccines also offer the potential for generating cell-mediated immune responses that could potentially provide cross-strain protection against different BTV serotypes. (Dunham , 2002; Dhama et al., 2008; Liu et al., 2006).

However, some small animal and clinical studies have indicated that "naked" DNA approaches are poorly immunogenic, due to physiological and cellular barriers that decrease transfection efficiency, including: nucleases; poor cellular uptake; poor translocation from the endosome to the cytoplasm; poor targeting to the nuclear membrane; poor translocation into the nucleus. When administered as an unformulated intramuscular injection, large quantities of DNA are required for mice (10-100μg), and larger animals (5–10 mg) to induce even modest immunogenicity (Anderson et al., 2007; Dunham , 2002). Other major areas of uncertainty exist regarding the design of suitable vectors and cytotoxic T cell responses, the vaccine delivery mechanism, and the amount of protein that is actually synthesised (Dhama et al., 2008). Indeed, the efficiency of translation of many viral genes that have been used in DNA vaccines is often suboptimal, thus limiting the amount of protein synthesised, and reducing vaccine immunogenicity (Dunham, 2002). The episomal nature of the plasmid DNA

vector, once it reaches the nucleus, also makes transfection transient, reducing immunogenicity (Anderson et al., 2007; Scheerlinck and Greenwood, 2008).

It is therefore, important for the success of any DNA-based vaccination or therapy, that a suitable route of administration is chosen to ensure efficient cellular uptake in the vaccinated animal. It is also important that the 'vector' for any vaccine provides a safe and efficient delivery system. This requirement has encouraged the use of non-viral gene-transfer techniques for DNA-mediated vaccines, these include liposomes, virosomes, microspheres and nanoparticles (Tyagi et al., 2008). The use of a gene gun significantly reduced the required dose of DNA for immunisation of mice, to 0.1-10ug. Intra lymphatic inoculation of 2ug of plasmid DNA induced an immune response greater than in those inoculated with 200ug of the same plasmid intramuscularly; suggesting differences associated with delivery (Dunham, 2002; Greenland and Letvin, 2007).

The study of DNA vaccines has made significant advances and a number of technologies aimed at increasing the potency of DNA vaccines are under evaluation in clinical or preclinical studies. These include improvements in the vectors themselves, the co delivery of cytokines, alternative delivery technologies, co induction of innate immunity. As DNA vaccines have the advantages of rapid production for large-scale distribution in the face of a pandemic disease or bioterrorist threats, there is urgency for the development of these enhanced vaccines (Liu et al., 2006)

The immune response generated by DNA vaccines is induced mainly via the professional APC (especially dendritic cells). These cells are primed by direct transfection with the plasmid DNA (direct presentation), or indirectly by the proteins produced by transfected somatic cells (cross presentation). Activated dendritic cells stimulate both MHC class I and MHC class II restricted responses, eliciting T-helper and cytotoxic T-lymphocyte (CTL), as well as B-lymphocyte responses. While proteins secreted from somatic cells or released from cells that have died as a result of apoptosis lead to activation of dendritic cells thus stimulating both MHC classes (Dunham, 2002) (figure 1.2). This apoptotic activity needs to be regulated, as too-rapid cell-death compromises antigen expression levels, while too low apoptotic activity may not promote cross presentation (Liu et al., 2006).

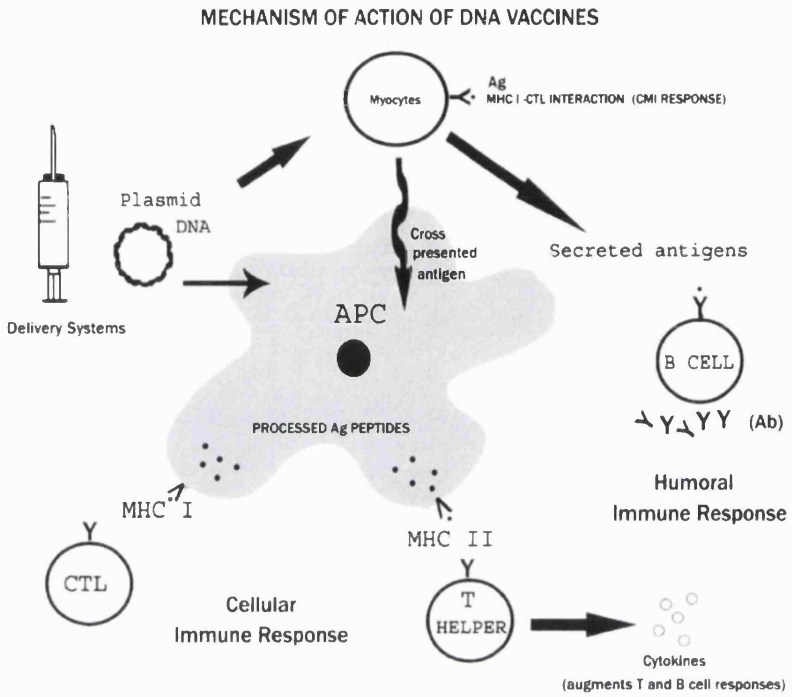


Figure 1-2 Generation of antigen-specific cellular and humoral responses by DNA vaccines

Professional APCs like dendritic cells and macrophages receive the secreted antigens (cross-presentation) from transfected somatic cells, or are directly transfected. They process and present the antigenic peptides to major histocompatibility complex (MHC) Class II molecules for helper T cells, which release a variety of cytokines to augment activation of cellular components of the immune system. Activation of cytotoxic T lymphocytes occurs by degraded antigenic peptides that are associated with MHC Class I molecules. These two mechanisms help in the generation of cellular immune responses. For humoral or antibody responses, B lymphocytes recognize and respond to antigens that are present extra-cellularly, or as secreted antigens (Dhama et al., 2008)

DNA vaccines often have low immunogenicity and their efficacy can be improved by exploiting other gene delivery methods, including use as a 'priming' vaccine, followed by a an alternative heterologous boost vaccination (such as a DNA/MVA – prime/boost - ref). The co-delivery of plasmids encoding biological adjuvants (Dunham, S. P., 2002; Dhama et al., 2008), and formulation agents that might be of specific importance in vaccination of target species have also been used to enhance the efficiency of naked DNA vaccines (Niborski et al., 2006). Determining the adjuvant used is also crucial for the type of immune response required.

1.8 : Recombinant vaccines

Advances in genetic engineering have made it possible to use a range of different 'vectors' for expression of unrelated genes as protective antigens (Moss, 1985; Mackett, 1987). The production of proteins which have only limited natural

availability has been enhanced in both Prokaryotic and Eukaryotic host systems by efficient transcription of cloned DNA and expression of heterologous proteins (Glover and Hames, 1995). This povides a basis for the development of future vaccines, that are potentially safe, inexpensive and multi serotype, producing long-term protective immunity after one 'shot' (Schwartz-Cornil et al., 2008). Several experimental recombinant vaccines have been described and they clearly have numerous potential advantages, over more conventional vaccines, including rapid onset of immunity, lack of transmissibility and even a polyvalent strategy (Savini et al., 2007).

The production of proteins which have only limited natural availability has been made possible in both Prokaryotic and Eukaryotic host systems by the development of a wide variety of vectors for efficient transcription of cloned DNA and expression of heterologous proteins (Glover et al., 1995). The recombinant protein production process consists of gene synthesis, cloning, expression and protein purification (Yokoyama, 2003).

1.8.1 : Protein Expression Systems

A crucial factor in obtaining recombinant expressed proteins in a native conformation and at the desired yield, depends on choosing an appropriate expression system (Brondyk, 2009). Four protein expression systems have been well described, including expression in: prokaryotic expression systems, yeast, insect cells, mammalian cells (Glover et al., 1995).The end products of these different systems can vary very significantly in protein stability, solubility, post-translational modification, functionality, timeline and cost considerations (http://www.genwaybio.com/gw_file.php?fid=6034).

The conformation / fold of the expressed polypeptides is determined by the host and expression system, and this can drammatically affect their antigenicity. However, the wide variety of expression vectors that are available for use in E. coli, has made these systems the most popular choices for producing recombinant proteins for immunological purposes (Glover and Hames, 1995).

1.8.1.1 : *E. Coli* expression

E. Coli is the prokaryotic system most frequently used for high level expression of heterologous proteins (Hannig and Makrides 1998). This is probably because its genetics are better charicterised than those of any other microorganisim, making it easy and inexpensive to use, with a high level of protein expression achieved within a

relatively short period of time (Baneyx, F. 1999). A yield of approximately 50-500mg/litre of culture can be achieved, making these systems attractive.

Analysis of large-scale protein expression trials show that up to 50% of proteins from the Eubacteria or Archaea and 10% of proteins from the Eukarya can be expressed in E. coli in a soluble form. suggesting that alternative systems should only be used after an *E. Coli* system has been reasonably explored (Graslund et al., 2008).

However it is also important to:

1) **choose a suitable strain of E. coli:** BL21(DE3) is commonly used for high level protein production purposes., with the advantage of being deficient in both *Ion* and *omp T* proteases, avoiding degradation of the the expressed protein (Glover and Hames, 1995). BL21 (DE3) has been used for a long time with pGEX4T2, even allowing expression of proteins that are toxic to the bacteria.

   This bacterial strain can tolerate large copy numbers of pGEX4T2, which contains a the high affinity tac promoter, resulting in high frequency transcription of foreign genes.

   2) Use an appropriate promoter: The site of transcription initiation is medited via interaction of transcription factors and their related promoter (Makrides, 1999). There are two classes of promoters, those that function constitutively and those that are regulated by inducers. Regulated propmoters are often useful for expression of proteins that could be toxic to the host. There numerous promoters ave been used for recombinant protein production in *E. coli* including the *lac* and T7 promoters. However, 'tac' is a strong IPTG inducable promoter, that allows accumulation of the expressed protein as 15-30% of the total cell protein content (Terpe, 2006).

   3) Use of affinity tags: These tags can have a positive impact on the yield, solubility, and folding, purification and subsequent use of their expressed-protein fusion-partner. However, there is no tag which is optimal in all aspects of protein production (Waugh, D. S., 2005). Most common tags such as β-galactosidase, trp E, bacteriophage T7, His6, maltose binding proteins and Glutathione-S–Transferase (GST), can be attached to either the N- or C terminus of the protein (Graslund et al., 2008; Yokoyama, S., 2003). Affinity

tags can be used for protein purification, and sometimes have a positive effect in the biochemical properties of the target protein, such as improving protein yield, preventing proteolysis, facilitating protein refolding and protecting the antigenicity of the protein with an increase in solubility. However these tags can sometimes also cause undesirable changes in the protein, altering conformation, reducing protein yield, inhibiting enzyme activity, altering biological activity, causing undesirable structural flexibility, or resulting in toxicity. Although it may be desirable to remove the tag after affinity purification (Arnau et al., 2006; Yokoyama, 2003), cleavage of the tag from the fusion protein may result in lowered solubility and/or stability.

The histidine residues in a His6 tag bind to divalent ions (Ni^{2}), allowing efficient purification of the tagged recombinant protein on nickel-chelate columns, (Glover and Hames, 1995). The small size of the His6 tag reduces the chances of a deleterious effects on protein characteristics (compared for example to GST). An N-terminal hexahistidine tag also helps to ensure that the bacterial transcription and translation machineries encounter a 5'-terminal sequence on the mRNA that is compatible with robust RNA synthesis and protein expression. For all of the above reasons, many researchers have selected N-terminal hexahistidine tag (Graslund et al.,2008). GST fusion proteins are often soluble, and are easily purifiable from E. coli cell lysates using glutation columns allowing the recovery of relatively pure proteins with high antigenicity (Glover and Hames, 1995).

4) Choose an appropriate Prokaryotic expression vector:

Vectors such as pET, pGEX and pMAL systems are generally recommended due to design advantages.

a) **pET:** all pET vectors contain the β-lactamase gene (confering ampicillin resistance), utilise the bacteriophage T7 RNA polymerase promoter and a leader sequence for efficient initiation of tranlation. They are designed to direct high level expression of cloned genes in DE3 lysogen strains of E. coli,. (Graslund et al., 2008; Studier and Moffatt, 1986; Glover and Hames, 1995).
b) **pGEX:** uses the induceble tac promoter, which directs expression of high levels of fusion proteins containing the glutathion S-transferase

(GST) gene fused to their carboxyl terminus. It has and unique restriction sites arranged to facilitate fusion of the insert DNA with the reading frame (Glover and Hames, 1995).

Although *E. Coli* is one the most commonly used expression systems, one of the drawback is that bacteria are unable to carry out post-traslational modifications that often occurr in Eukaryotic sysytems (Brondyk, 2009). Another drawback is that most efficiently expressed proteins in bacteria are directed to the cytoplasm, often leading to aggregation of incompletely folded and insoluble proteins in the form of inclusion bodies (IB) (Terpe,.2006; Glover and Hames, 1995). However, recent studies indicate that the fraction of properly folded and biologicly active proteins that are present in IBs, can be enhanced by growth at low temperatures (Vera et al., 2007).

1.8.1.2 : Expression of cloned genes in Yeast

This can be achieved using *Pichia pastoris* and *Saccharomyces cerevisiae* and has been used for expression of human genes (Graslund et al., 2008; Yokoyama, S., 2003). These expression systems are simple and inexpensive to use, although, their expression level is variable (at 10-200mg/liter of culture) and is lower than frequently obtained in E. coli. However, yeast systems are often regarded as the first eukaryotic system to try if expression in E. coil is unsuccessful (Glover and Hames, 1995).

1.8.1.3 : Insect cells (Baculovirus expression system)

Bauloviruses constitute one of the largest and most diverse groups of viruses, with the baculovirus disease of insects first described in 1527 as jaundice disease of the silk worm Bombyx mori (Glover and Hames, 1995). The Baculoviruses (family *Baculoviridae*) belong to a diverse group of large double-stranded DNA viruses. They are highly species–specific, infecting many different insects, but are not known to propagate in any vertebrate host . These insect-cell-based protein production systems have many advantages for VLP production, including:

a) Large amounts of recombinant proteins can be produced in eukaryotic cells, in high-density cell cultures, allowing efficient recovery of correctly folded antigen.

b) As the insect cells used to support baculovirus replication for vaccine production, can be cultured without mammalian-cell-derived supplements, the risk of culturing opportunistic pathogens is minimised.

c) The baculoviruses used for recombinant protein expression have a narrow host range that includes only a few species of Lepidoptera and do not therefore represent a major threat to vaccinated individuals or risk of environmental damage.

d) Baculoviruses are easily inactivated by simple chemical treatments and are localised mainly in the nucleus, while most VLP are purified from cytoplasmic extracts.

e) Baculoviruses are able to incorporate relatively large segments of additional foreign DNA sequences with little effect on replication efficiency or DNA packaging within the nucleocapsid. The largest reported insert is approximately 15Kb.

f) Baculovirus infection of *Spodoptera frugiperda* cells results in the shut-off of host gene expression allowing for a high rate of recombinant mRNA and protein production (manual)

g) Perhaps most importantly the baculovirus system can be scaled-up for large scale vaccine- production (Noad and Roy, 2003; Belyaev and Roy, 1993; Glover and Hames, 1995).

The ability to express multiple genes simultaneously, with relatively simple purification methods, helps explain why this system has been used extensively for the expression of recombinant proteins and production of both VLP and CLP of BTV for vaccine development (Roy 1992b; Van Dijk, 1993; French et al., 1990; Roy et al., 1990; Roy, 1990). Baculovirus expression has also been used for the development of diagnostic reagents and materials for X-ray crystallography studies, helping to provide a better understanding of the structure of the virion and the assembly process of this architecturally complex particle (Roy, 1990; Luo and Sabara, 2008; French and Roy, 1990; Roy et al., 1997; Wang et al., 1999; Hassan and Roy, 1999; Hassan et al., 2001; Hewat et al., 1992).

The most popular baculovirus used for recombinant expression is multiple virion nuclear polyhedrosis virus (MVNPV). In this case multiple virus particles become occluded within individual nucleus-located protein crystals, known as polyhedra. Because of its ease of handling in tissue culture, MVNPV from *Autographa californica* (AcNPV) the Lepidopteran alfalfa looper, are used. The ovarian cell line S*f*9, derived from *Spodoptera frugipedra* pupae, is most frquently used and is highly recommended for the propagation of (AcNPV) and its recombinants (Glover and

Hames, 1995). In this system several Baculovirus genes that are non-essential in the tissue culture life cycle of the virus (e.g. polyhedrin, p10, basic) can be replaced by heterologous genes. Since the baculovirus genome is generally too large for easy manipulation to insert foreign genes, heterologous genes are initially cloned into transfer vectors. Co-transfection of the transfer vector and AcNPV DNA into *Sf* cells allows recombination between homologous sites, transferring the heterologous gene from the vector into the AcNPV DNA.

1.8.1.4 : Mammalian cells

These are considered the least efficient vehicles for recombinant protein expression but can be used for stable or transient expression, with Chinese hamster overy cells (CHO) and Human embryonic kidney cells (HEK293) being the most commonly used cells (Brondyk, 2009).

1.8.1.5 : Viral delivery systems (Recombinant viruses)

Recombinant live viruses expressing foreign antigen have been used widely as a delivery systems (vaccine candidates) for infectious diseases and cancer (Wang et.al 2010). These include: Adenovirus, Corona virus, Herpes Simplex virus, Lentiviruses, Poliovirus, Retrovirus, Vaccinia virus. Viral vaccines have the advantage of stimulating both the humoral and cellular immune response to the target antigen. This is believed to be due to the intracellular expression of the heterologous antigen, leading to presentation by the MHC class I molecules (Liljeqvist and Ståhl 1999).

1.8.1.6 : Poxviruses as a delivery vectors

Several poxviruses have been used successfully as vaccine delivery vectors. These include the Copenhagen strains of vaccinia virus (used to express the Rabies virus glycoprotein), fowlpox based recombinants (expressing Newcastle disease) and recombinant canarypox virus used to protect against rabies, canine distemper, feline leukaemia and equine influenza viruses (Esteban 2009)

Recombinant Vaccinia, recombinant Canarypox virus, recombinant capripox virus have all been used successfully as gene delivery system for BTV-vaccination (Boone, 2007; Wade-Evans et al., 1996; Savini et al., 2008).

a) Vaccinia virus

Vaccinia virus is a large DNA virus that replicates in the cytoplasm of a wide range of vertebrate host cells. It is a prototypic member of the genus *Orthopoxvirus,* family

Poxviridae (Moss and Earl, 1998; Falkner and Dorner, 2005). It is also one of the most widely applied expression systems used and has successfully helped in smallpox eradication. The protection induced by immunisation with recombinant vaccinia virus is mostly mediated by $CD8^+$ T cells (Li et al., 1993).

The expression of heterologous genes in a vaccinia virus system requires integration of the foreign DNA into the vaccinia virus genome by means of homologous recombination, or by direct molecular cloning. In both cases, plasmid vector constructs are required that contain the gene of interest and usually a marker gene, both of which are controlled by suitable promoter sequences (Pfleiderer et al., 1995). The level of gene expression is regulated by the choice of an early, intermediate, or late promoter If large amounts of target protein are desired then a promoter with high transcriptional activity should be used. The highest levels of expression have been obtained with strong natural or synthetic late or early/late promoters (Moss B., 1998)

Vaccinia has capacity as a vector that permits cloning of large fragments of foreign DNA (>20K bp) with retention of infectivity (Moss and Earl, 1998). This helps explain why recombinant vaccinia viruses have been widely used and are important for immunological studies (Moss, 1985). Since this system produces relatively high level of protein, and has a high success rate for production of soluble and functional protein, with a yield of (0.1-100mg/liter of culture) (Moss and Earl, 1998; http://www.genwaybio.com/gw_file.php?fid=6034).

Methods for the production of recombinant poxviruses employ plasmid transfer vectors, containing an expression casette, consisting of a poxvirus promoter with adjacent restriction endonuclease sites allowing insertion of the foreign genes. The expression cassette is flanked by vaccinia virus DNA sequences that direct recombination into the desired locus by homologous recombination. Screening is provided by targeting the foreign gene to the TK locus. Recombinant viruses can be then be selected by their TK-negative phenotype in TK-deficient cells. Alternatively, the transfer vector may contain a reporter gene allowing colour screening e.g. due to B-galactosidase synthesis ((Moss and Earl, 1998)

Recombinant vaccinia virus has been used to express BTV VP2 and VP5 as individual proteins in a double recombinant, or with the core protein (VP7) as a triple-recombination (Lobato et al., 1997; Savini et al., 2007). Highest protection was obtained in groups immunised with both proteins (VP2 and VP5), which stimulated

anti BTV antibodies (Lobato et al., 1997; Boone et al., 2007). However, this system has not been widely used for the production of BTV-VLP for vaccination (Noad and Roy, 2003). In some cases, this could be due to the relatively low yield of protein (0.1mg/litre) and a relatively high cost of the system (Pfleiderer et al., 1995). To increase safety, a highly attenuated and non-replicating vector has been developed called Modified Vaccinia Ankara (MVA), which was obtained by serial passage in chicken embryo fibroblasts, resulting in strains that are no longer able to grow in mammilian cells (Falkner and Dorner, 2005; Anderson et al., 2007).

i) Modified Vaccinia Ankara (MVA)
Chorioallantois vaccine virus Ankara (CVA)] was passaged over 570 times in primary chick embryo fibroblast cells (CEF), leading to attenuated of its replication and virulence. This resulted in formation of a new virus strain that is named Modified Vaccinia Ankara (MVA). MVA has major deletions in its DNA, totalling 31,000 base pairs (bp), and although it still directs unimpaired synthesis of both viral and recombinant protein in mammalian cells, its own replication is blocked late in morphogenesis (Sutter and Moss 1992). However, MVA can be propagated in CEF, and easily maintained in cell line such as BHK cells. This has improved accessibility of MVA for the development of expression vectors and live recombinant vaccines (Drexler et al., 1998)

MVA has shown real promise as a vaccine vector, and already has a history of use as a vaccine for infectious diseases and malignancies, allowing high expression of the gene of interest with a well established safety record. MVA is also very stable, giving vaccines a long shelf-life and gives good immunogenicity in the absence of adjuvants, activating both branches of the immune system (Esteban 2009; kennedy and Greenberg 2009; Sutter and Moss 1992; Ramirez et al., 2000).

These factors have made MVA an attractive delivery vector, that has previously been used succesfully (and in the study described here) for the expression of orbivirus proteins (of both BTV and AHSV) and subsequent vaccination trials (Calvo-Pinilla et al., 2009; Castillo-Olivares et al., 2011)

b) Recombinant Canarypox virus: Boone, (2007) developed a recombinant canarypox virus expressing outer-capsid proteins VP2 and VP5 of BTV-17. This appeared to safely and effectively induce protective immunity to Bluetongue in sheep.

c) Recombinant capripox virus:
A recombinant capripox virus expressing VP7 of BTV-1 was shown to induce antibodies to VP7, but no neutralising antibodies to either the homologous or heterologous serotype (BTV -3) (Wade-Evans et al., 1996; Savini et al., 2007). However, it did provide partial protection against heterologous BTV challenge.
Perrin et al., (2007) used (BTV-Cpox) individually expressing four different genes, encoding two capsid proteins (VP2 and VP7) and two non-structural proteins (NS1, NS3) for vaccination trial in sheep. Partial protection was provided in the sheep when challenged with a virulent strain of BTV-2 three weeks post vaccination.

1.9 : Adjuvants

While subunit and DNA vaccines can potentially be produced safely and reproducibly in bulk. They do not replicate in the vaccinated animal and therefore can have relatively low immunogenicity, compared to attenuated strains or recombinant virus vectors (Bramwell et al., 2005; Aucouturier et al., 2001). It is therefore important that they are administered in an appropriate "adjuvant format" to maximise the immune response (Bramwell et al., 2005).

Many adjuvants are simple compounds that are mixed with the vaccine-antigens in the later stages of production, relying on a 'depot effect' to stimulate their immunogenicity (Hughes, 1998). However, there is no universally accepted 'best' adjuvant and the materials used must be adapted according to several criteria. These depend on the target species, the antigen, the route of inoculation, the type of immune response and the duration of immunity required (Aucouturier et al., 2001). In a review of particulate delivery systems for animal vaccines, Scheerlinck and Greenwood, (2006) emphasise that adjuvants should be designed to provide a targeted immune response, with little or no side effect. The ease of delivery of slow release systems, is specially important in providing a continuous supply of antigen over weeks or months, which will ultimately 'boost' the immune response. Encapsulation of antigen in some preparations, in order

to protect it from degradation, may be especially effective in single dose administration.

1.10 Vaccine production regulations

The generation of veterinary vaccines has many factors in common with the development of human vaccines. These include a need for protective (preferably sterile) immunity in the majority of (preferably all) vaccinated animals, coupled with minimal side effects, ease of handling and administration.

The great diversity of animal species that can be affected even by a single virus strain (e.g. BTV in different ruminants, camelids and carnivores), indicates a requirement for generic vaccine and adjuvant systems that will work across different species (Scheerlinck and Greenwood, 2006). Vaccines must not present any unacceptable risks to target animals, the environment, consumers of food from animal origin, animal handlers, the public, or the users of the vaccine product. They therefore need to be accepted in accordance with established criteria, such as those set out for the European market (Alpar et al., 2008). The regulations that are currently in place for live attenuated BTV vaccines, include: sterility tests for the presence of viable bacteria, fungi and mycoplasma; safety tests, where a batch sample is administrated to new born mice, adult mice, guinea pigs and sheep; potency checks, including duration of immunity; and stability over a set period of time (OIE, 2004; Saegerman et al., 2007).

1.11 : Bluetongue infection and the host immune response

Immunological studies of BTV have indicated that protection against infection can involve components of both the humoral and cellular immune response (Jeggo and Wardley, 1985; Andrew et al 1995). Animals can be at least partially protected against BTV infection and severe clinical signs, by a cell mediated respose, in the absence of neutralising antobodies. These responses are also frequently cross-protective between serotypes. However, all animals that develop a strong neutralising antibody respose are protected against infection and clinical signs caused by the homologous BTV serotype.

1.11.1 : The Humoral Immune response to BTV

BTV-serotype-specific neutralising antibodies can be detected in sheep at around 10-12 days post infection, and in cattle at around 10-20 days post infection.The response following infection by a single BTV serotype is monotypic, with neutralising antibody titres remaining high for more than 7 months (Jeggo et al., 1983b). This provides protection when animals are challenged with a BTV strain belonging to the same serotype. When serial infection with different serotypes, sheep developed heterotypic neutralising antibody respose to a number of serotypes (Jeggo et al., 1983a).

The neutralising antibody response is directed primarily against BTV outer-capsid protein VP2, is serotype-specific (Jeggo and Wardley, 1985; Maan et al., 2007a). However, cross-reactive (serogroup-specific) antibodies are also generated against the more conserved viral proteins, particularly immunodominant outer core protein VP7(T13) (Gumm and Newman, 1982). Maclachlan et al., (1987) studied the humoral immune response of colostrum-deprived calves to bluetongue virus showing that virus-specific neutralising-antibodies developed to VP2 (the major determinant of virus neutralisation) at 14-28 days post infection. The development of homologous and heterologous neutralising antibodies depended on whether animals are experimentally inoculated simultaneously or sequentially with different BTV serotypes (Dungu et al., 2004). Passive serum transfer studies demonstrated serotype specific protection in sheep, thus indicating the importance of neutralising antibodies in protection (Jeggo et al., 1984), however the level of neutralising antibody does not always correlate with protection (Schwartz-Cornil et al. 2008).

1.11.2 : Interferon (IFN)

Interferons are an extraordinary group of proteins, whose antiviral activity led to their discovery almost 50 years ago (Kurby, 2000).

Three families of IFNs have been identified: type I IFNs (including IFN-α, IFN-β, and IFN-ω) and type II IFN (IFN-γ), which are structurally unrelated (Muller et al., 1994) and recently descrived type III IFN (IFN-λ)(Vilcek, 2003).. Interferons IFN-α and IFN-β are secreted from macrophages and fibroblasts respectively, and induce an antiviral state in most nucleated cells, increase MHC class I expression and activate NK cells (Kurby, 2000)

Virus associated molecules, such as dsRNA, that are produced in virally infected cells can be recognised by pattern-recognition receptor (PRRs), resulting in an effective and appropriate antiviral response. Type I IFNs (IFN-α IFN-β) are the main cytokines

produced after a viral infection mediating and inducing both innate and subsequently an adaptive immune response (Kawai and Akira 2006).

Because BTV is double a stranded RNA virus, it is a strong type I IFN inducer in sheep, cattle and mice (Jeggo and Wardley, 1982b; Foster et al., 1991; MacLachlan et al., 1985). These authors first detected type I IFN in serum samples from sheep infected with several different BTV strains, on day 5(±1), reaching a peak on day 6(±2). This coincided with the first peak of viraemia. The IFN peak concentration induced a 90% decrease in virus titre. The IFN concentration had decreased by day 9 (±2), corresponding with a second peak of viraemia which in turn corresponded with the initial detection of serum antibodies to BTV on day 10 (±2).

Mice lacking a subunit of the type I interferon (IFN) receptor are completely unresponsive to type I IFNs, suggesting that this receptor chain is essential for type IIFN-mediated signal transduction (Muller et al., 1994). These mice showed no overt anomalies but were unable to cope with viral infections, despite otherwise normal immune responses (Muller et al., 1994). The lack of an IFN system allows the virus to replicate more efficiently and IFNAR(-/-) mice were established as a new laboratory animal model suitable for the evaluation of vaccination strategies against BTV (Calvo-Pinilla etal., 2009a).

1.11.3 : The Cell mediated immune response

Cytotoxic T-cells (CTL) have the ability to destroy virus infected cells early in infection, helping to prevent viral spread (Jeggo and Wardley, 1982a; 1982b; 1982c). BTV induces murine cytotoxic T lymphocytes (CTLs), which are at their maximum activity at around day 7 post infection (p.i), and are detectable for around three weeks. In sheep CTLs peak at around day 14 p. i. and are detectable for 2 ½ weeks following virus inoculation (Jeggo and Wardley, 1982a; 1982b). These cells are cross-reactive between different BTV serotypes, giving rise to 'heterologous' protection, although this is relatively short lived (Jeggo and Wardley, 1985). This was also observed in sheep by (Jeggo et al., 1984), who adaptively transferred thoracic duct lymphocytes from sheep that were at 14 days post infection with BTV-4, to uninfected monozygotic sheep, which were subsequently protected when challenged with BTV-3. Although CTLs do not prevent virus infection, they can act to clear the homologous or heterologous BTV serotypes from an infected host (Jeggo et al., 1984). Before embarking on vaccine development and production it is important to determine the

immune response that is required for protection and identify the antigens necessary to produce that response (Jeggo and Wardley, 1985). A number of CTL epitopes recognised by sheep were identified within NS1, VP2, VP3, VP5 and VP7 (Janardhana et a., 1999).

Helper T cells are essential for effective antibody response, induction of high affinity neutralising antibody and Ig class swiching. The major BTV serotype specific helper T cell epitopes recognised by sheep are identified with VP2, some in VP5 and serotype-crossreactive helper T cell epitopes in core proteins (Takamatsu et al., 1990; 1992; Jones et al., 1996).

1.12 : The immune response following Bluetongue vaccination

1.12.1 : Killed BTV vaccine

Stott et al., (1985) studied the humoral and cellular immune response in sheep vaccinated with inactivated BTV. In these experiments all of the vaccinated animals developed BTV group-specific non-neutralising antibodies, with some variability depending on BTV strain / serotype and sheep breed, as well as some individual variations, but no neutralising antibodies were detected. This could be due to the affect of the binary ethylenimine (which is a bifunctional cross-linking agent) used in inactivation, which can alter some antigenic characteristics of VP2 (the viral protein targetted by neutralising antibodies) (Odeon et al., 1999). However, neutralising antibodies were developed post-challenge,, the titre of which correlated with the level of protective immunity (Stott et al., 1985). Similar results were obtained in cattle that were inoculated with inactivated BTV vaccines and boosted 3 weeks later (Odeon et al., 1999).

Inactivated BTV vaccines also failed to induce a CTL response in a murine model (Jeggo and Wardley, 1982a), suggesting that replicating virus was required to induce a primary CTL response. In later studies NS1 was shown to be one of the major immunogens for CTL (Andrew et al., 1995). This protein forms tubules in infected mammalian cells (Roy, 1992a; Anderson et al., 1993). Anti tubule antibodies are detected approximately 10 days post-infection in BTV infected sheep. However, these antibodies are not detected in sheep injected with an inactivated BTV vaccine. Although this may not be consistent with other inactivated vaccine preparations, anti-tubule antibody could potentially be used as a valuable mean of discriminating infected

sheep from those vaccinated with inactivated BTV that do not contain NS1 (Anderson et al., 1993).

1.12.2 : Live attenuated BTV vaccines

The ability of attenuated BTV strains (generated via serial passage in tissue culture or in embryonated chicken eggs) to replicate in the ruminant host (e,g. cattle, goats or sheep) allows them to generate a strong antibody response (Savini et al., 2007). Unlike killed vaccines they are also able to induce CTL (Dungu et al., 2004). NS1 and VP2 proteins appear to be recognised most frequently by CTL, with both proteins containing more than one CTL epitope. The use of multivalent, whole, live-virus vaccines allows a greater and more extensive induction of the CTL response, due to the presence of multiple CTL epitopes (Dungu et al., 2004).

1.12.3 : DNA vaccines

These vaccines can be used to induce antibody, CD4+ helper T cell responses, as well as CD8+ CTL responses. Following intramuscular inoculation of the plasmid DNA, the gene products expressed by muscle cells are phagocytised by antigen presenting cells. They are either cross-presented on MHC class I molecules inducing CD8+ CTL responses (because they express antigens intracellularly) or are presented by MHC class II molecules, inducing CD4+ helper T cell responses. This combination of co-stimulation with MHC classes I and II presentation of antigen leads to the initiation of immune responses mimic natural virus infection (Anderson et al., 2007; Greenland and Letvin, 2007). No BT DNA vaccines are currently available.

1.12.4 : Subunit vaccines

VLP can efficiently stimulate both cellular and humoral immune responses, based on efficient stimulation of MHC class I and MHC class II. (Noad and Roy, 2003). VLP containing VP2, generate neutralising antibody, protecting sheep from homologous BTV challenge (Roy et al., 2003), Although VP2 was poorly immunogenic in rabbits (Lobato et al., 1997), the combination of VP2 and VP5 provided the most consistent protection for sheep (Lobato et al., 1997). This correlates with the results obtained by (DeMaula et al., 2000) where amino acid substitution in VP2 was responsible for neutralisation-resistance in most escape-mutant viruses of (BTV-10). However, in one case an amino acid substitution in VP5 (with no change in VP2) also resulted in a neutralisation resistance escape-mutant virus (EMV). This confirms that VP2 contains the major neutralisation determinants of BTV, while VP5 can influence neutralisation

of the virus. Similar conclusions were drawn from studies using reassortant viruses (Mertens et al., 1989; Cowley and Gorman, 1989).

It has also been possible to stimulate heterologous protection using VP2, indicating that either a cross-reactive cell mediated immunity exists, or antibodies to shared epitopes on VP2 can protect (Lobato et al., 1997). However, the level of neutralising antibodies does not always correlate with the degree of protection and some inactivated vaccines preparations can provide protection in the absence of detectable levels of neutralising antibodies (Schwartz-Cornil et al., 2008 Takamatsu and Jeggo (1989) showed that most of the BTV specific T cell lines prepared from BTV immune sheep, responded to both homologous and heterologous serotype viruses replicating in skin fibroblasts. This provides evidence of a cross-protective immune response to BTV that involves CTL (Schwartz-Cornil et al.,2008). BTV VP2 and NS1 are major immunogens involved in the CTL response, while VP5 and NS3 are minor immunogens and no specific CTLs were detected targeting VP7. (Stott et al., 1985; Dungu et al., 2004; Andrew et al., 1995). However, this contradicts results obtained by Janardhana et al., (1999), where BTV-1 VP7 protein expressed in (vaccinia virus expression system) was recognised by CTL in some Australian merino sheep. NS1 was also shown to induce a CTL response s in mice (Ghosh et al., 2002).

BTV VLP provide an excellent delivery mechanism for BTV epitopes, probably because particulate VLP are taken up by antigen–presenting cells. This stimulates a long lasting CTL response, an antibody response (Noad and Roy 2003) and a CD4+ lymphocyte proliferation response. They therefore represent potential candidates for next generation nsubunit vaccines, although their cost of production, purification and poor stability may limit their wider application in the field.

1.13 : Aims and Objectives

The overall aims of this project was to generate reagents and strategies for a safe and effective, vaccination against BTV serotype-8 and assess the protective immune response by production of neutralising antibodies in vaccinated mice. We anticipated that the results of this project would have applications in the fight against the BTV outbreaks in Europe, and potentially elsewhere, that may make it an attractive target for commercialisation. One of the primary goals was based around expression of BTV-8 major viral proteins in *E. Coli* It was considered possible that the expression of the

major viral protein, VP2 fragmented while overlapping would stimulate a neutralising immune response in mice. This hypothesis was investigated by analysing the neutralising antibodies stimulated post vaccination with fragmented VP2.

This project also aimed at identifying the efficiency of in vivo expression of the major viral proteins for protecting from challenge in comparison to that following vaccination with recombinant proteins expressed in bacteria.

The specific aims of this project therefore included the following:

1. Generate cDNA copies of BTV genome segments 2, 6 and 7 from a European BTV strain (BTV-8)
2. Clone BTV ORFs, for expression of proteins VP2, VP5 and VP7 into plasmids for expression and vaccine studies.
3. BTV proteins expression by bacteria (particularly outer capsid proteins) for vaccination studies.
4. Develop DNA vaccination strategies for BTV using plasmids generated by objective 2 and Immunogenicity profiles to be tested in a small animal model
5. Generation of recombinant MVA vaccinia virus to express BTV proteins (VP2, VP5 and VP7), for vaccination studies
6. Evaluate purified bacterial expressed proteins, combinations of rMVA expressing BTV proteins or DNA for development of vaccines in a small animal model. The possibility of inducing protection from clinical signs
7. Evaluate the neutralising antibody response, after vaccination with DNA, recombinant MVA, recombinant BTV proteins, either individually, or in combination (with and without field-relevant adjuvants) in a small animal model (mice)
8. Identify the efficiency of VP2 alone in protecting mice from a lethal dose of the infectious virus.
9. Providing a vaccine that is compatible with a DIVA assay.

2 CHAPTER 2
Materials and Methods

2.1 Materials and Methods

2.2 Viruses

2.2.1 Virus propagation in BHK-21 cells

The different virus strains used were obtained from the dsRNA virus collection at IAH Pirbright (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm). BTV isolates were propagated in BHK-21 cells (maintained at IAH, Pirbright) in 175 cm^2 tissue culture flasks. The cells were grown and maintained in Glasgow medium, containing 5% foetal calf serum. The western BTV isolates used included (BTV-4, GRE2000/07; BTV-9, GRE1999/06; BTV-16, GRE1999/13; BTV-8, NET2006/04 and BTV-8, NET2007/08), while the eastern BTV isolates included (BTV-12, BRA2002/01; BTV-20, AUS1975/01 and BTV-21, AUS1979/02). For BTV infection, the growth medium was removed from a BHK cell monolayer (75cm^2 flask) which was then incubated with 5 ml of virus (supernatant from infected cell cultures, at a dilution of 1:10 in eagles medium without serum) for 30 min at RT, to allow virus adsorption and internalisation. Subsequently 50 ml of maintenance medium (Glasgow medium containing 2% TPB) was added to each flask. Flasks were then incubated at 37°C until full cytopathic effect (CPE) was observed (usually 24-48 hours). Cells showing 100% CPE were harvested and centrifuged at (1200 xg) for 5-10 minutes, to pellet the cell debris. Supernatant and cell debris were stored at 4°C.

2.2.2 Generation of MVA virus stocks

T75 flat tissue culture flasks of DF-1 (a chicken fibroblast cell line obtained from the Institute for Animal Health Central Service Unit (CSU) were infected with MVA at moi 0.01, for 1 hour at 37°C in serum free medium. The inoculum was removed and 15ml/flask of DMEM is added (containing 2.5% foetal calf serum). Flasks were incubated for 3-4 days until 80-100% cytopathic effect (cpe) is visible. Cells were scraped from the flasks and the cell suspension pelleted at 1500 x *g* for 10 minutes. The pellet was resuspended in 1ml serum free DMEM and freeze-thawed three times to lyse the cells. The final stock was then titrated using a sensitive X-Gal staining plaque assay, which will help to confirm the purity of the MVA virus clone.

2.3 RNA purification

2.3.1 : RNA extraction using TRIzol®

dsRNA was extracted from virus-infected cell cultures, using a guanidinium isothiocyanate procedure and reagent commercially known as TRIZOL (Gibco BRL), as previously described (Attoui et al., 2000). Briefly, BTV infected cells were pelleted by low speed centrifugation (1200 x *g* for 10 min). Most of the virus is cell associated and the supernatant was therefore poured off into disinfectant and the cell pellet retained. 1ml of Trizol was added to each cell pellet, with vigorous shaking until the pellets dissolved. 200µl of chloroform was added, the mixture shaken for 1 min, incubated on ice for 10 min, then centrifuged at (12000 x *g* at 4°C). The aqueous phase was recovered using a pipette and transferred to a 1.5 ml tube containing 900 µl of isopropanol then incubated at -20°C for 2 h. The mixture was centrifuged at 18000 x *g* for 10 min at 4°C. The RNA pellet was recovered, washed with 1 ml of 70% ethanol, air dried, and then dissolved in 100 µl of RNase-free water. ssRNA was removed by mixing the RNA preparation with an equal volume (100µl) of 4 M LiCl solution, incubating overnight at 4°C and centrifugation at 18000 x g for 1 min. The dsRNA in the supernatant was then precipitated by adding 200µl of isopropanol and 50µl of 7.5M ammonium acetate, followed by incubation at -20°C for 2 hours, then centrifugation at 18000 x *g* for 10 minutes at 4°C. The pellet was washed with 1 ml 70% ethanol and air dried, and then dissolved in 50µl RNase free water. The quality of the dsRNA was assessed by 1% agarose gel electrophoresis (AGE) (7 V/cm, for 1 h) in TAE buffer containing 0.5µg/ml of ethidium bromide.

2.3.2 : RNA extraction from cell sheets using the Qiagen RNeasy mini Kit

The supernatant was first removed and discarded, from CEF cell sheets grown in 12 well plates (described in section 2.20). The cells were then lysed in 350 µl of Buffer RLT (provided with the kit and containing added β-ME), collected into a 1.5 ml microfuge tube and disrupted/homogenised by vortexing for 1 minute. Lysates were then subjected to the 'animal cells spin' protocol of the RNeasy mini kit. Briefly, 350 µl 70 % ethanol was added to the lysate, mixed by pipetting and bound to the spin column membrane by centrifugation at 15,000 *g* for 30 seconds. The membrane was washed once with 700 µl of Buffer RW1 (provided with the kit), and twice with 500 µl Buffer RPE (provided with the kit and diluted with 96-100 % ethanol); centrifuged at 15.000 *g* for 30 seconds. The RNA was eluted from the column by two rounds of

centrifugation at 15.000 *g* for 1 min, each time using 40 µl of RNase-free water. RNA was used immediately for one–step RT.

2.3.3 : Robotic RNA extraction from blood samples

S-block 96 well, deep well plate was loaded with 40µl/well of Protease (BIOrobot MDXkit) reconstitute Qiagen Protease with protease solvent. Blood samples treated with EDTA (50µl, obtained from challenged mice at various dates post challenge), were loaded onto respective wells. In the same manner 50µl of positive and negative controls were distributed throughout the plate. This was followed by the addition of 190µl of Nuclease free water and 360µl of Roche lysis buffer. RNA was extracted from this mixture on a MagNA Pure LC robot (Roche) using the protocol 'total NA/External_lysis' according to the manufacturer's instruction.

2.3.4 : Full-length amplification of cDNAs and anchor-primer ligation

The method for full-length amplification of complementary DNA (FLAC) was previously described (Maan et al., 2007). The use of a self priming 'anchor-primer' helps to ensure full-length first-strand cDNA synthesis in the absence of free floating primers, preventing any non-specific amplification and mis-priming.

a) Anchor-primer ligation

A 35 base oligonucleotide 'anchor-primer' (5'p-GACCTCTGAGGATTCTAAAC /iSp9/TCCAGTTTAGAATCC-OH3') was ligated to approximately 500 ng of unfractionated viral dsRNA, as described by Maan et al (2007). Ligation reactions were carried out in a total volume of 10 µl, containing: dsRNA 1 µl (500–1000 ng), anchor-primer 1 µl (500 ng), 10X T4 RNA ligase buffer 1µl (New England Bio Labs) and 10U T4 RNA ligase (New England Bio Labs) and incubated at 4 ◦C for 12 h.

b) Electrophoretic separation of ligated RNAs and re-extraction from the gel

After ligation to the anchor primer, the dsRNA genome segments were separated by 1% agarose gel electrophoresis (AGE) in TAE buffer. Genome segments that co-migrated during AGE were recovered as mixed preparations (e.g. seg-2 and 3). Correctly sized bands were excised using a fresh scalpel blade, and recovered using the RNaid kit (BIO 101) as previously described (Attoui et al., 2000). Each agarose slice was melted at 55°C in 3 vol (300 µl) of the RNA binding salt solution. 10 µl of the RNA matrix (silica beads) was added, the mixture was mixed thoroughly then incubated for 10 min at room temperature, to allow binding of the RNA to the matrix. The mixture was centrifuged for 1 minute in a microcentifuge at maximum speed to

pellet the RNA/RNaid-Matrix complex. The supernatant was discarded and the pellets were washed 3 times with 500 µl of RNA wash solution, with centrifugation for 1 minute in a microcentrifuge at maximum speed. The supernatant was discarded on each occasion. The final pellet was resuspended in 10µl RNase-free water and RNA was eluted from the matrix by incubating at 55°C for 5 minutes. The suspension was then transferred to a SPIN Filter and centrifuged for 1 minute in a microcentrifuge. The supernatant containing RNA was recovered from the catch tube and stored at −20 ◦C or used immediately for cDNA synthesis.

2.4 : cDNA first strand synthesis (Reverse Transcription RT)

11 µl of RNA solution was denatured by boiling for 3 min. The mixture was immediately quenched in an ice-water bath to prevent re-annealing of the dsRNA. Nine microlitres of a 'Reverse Transcription mix' was added, containing 4µl of 25mM $MgCl_2$, 2µl of 10mM each dNTP mix, 15 U of AMV reverse transcriptase, 20 U of RNAsin and 2µl of 10X RT buffer (Reverse transcription System, Promega). The reaction was incubated at 37 °C for 40 min, then at 40 °C for 10 min. The cDNA was used directly for PCR amplification or stored at −20 °C.

2.5 : Sequencing Reactions

2.5.1 : Sequencing of PCR amplified cDNA segments

The sequence of the terminal 40–50 nucleotides from either end of the genome segment were often unreadable using terminal primers. Two outward-facing near-terminal primers (forward and reverse 'foot-print' primers (FppF and FppR) were therefore used to provide the sequence of these near terminal regions. Full-length BTV genome segments were sequenced (with overlaps) using the 'Cycle Sequencing Ready Reaction' kit (CEQ DTCS Beckman Coulter) on a Beckman Capillary Sequencer. Sequencing reactions contained: 50ng of DNA template, 3.2pmols primer, 8µl Quickstart mastermix (Beckman CEQ) and nuclease free water to a final volume of 20µl. The sequencing reactions of PCR products were incubated for 30 successive cycles as follows: 96 °C for 20 seconds, 50 °C for 20 seconds, and 60 ° C for 4 minutes. The reactions were subsequently precipitated with ethanol (according to the Beckman protocol) resuspended in 40 µl of sample loading solution and run on a Beckman sequencer (CEQ8000). The primers were used for sequencing segment 2 and 6 of BTV-4, BTV-9 and BTV-16 are shown in Table 2.1 to Table 2.6:

Primer designation (aa)	Primer sequence (5'-3')
BTV-4/2/Fpp106R	TTCATCCACTTAGCATCCGTC
BTV-4/2/p106F	GACGGATGCTAAGTGGATGAA
BTV-4/2/p233F	GGTCTATCATATAACAGATT
BTV-4/2/p474F	GTGTAAGATAGATGATGAG
BTV-4/2/p841R	AACTTGGACGTCACAACAGG
BTV-4/2/p841F	CCTGTTGTGACGTCCAAGTT
BTV-4/2/p876R	TGCGGGTCGAGCTTATC
BTV-4/2/Fpp876F	GATAAGCTCGACCCGCA

Table 2-1 BTV-4 segment-2 (Seg-2) sequencing primers

Primer designation (aa)	Primer sequence (5'- 3')
BTV-9/2/eup245F	ATAGATATGAGCGATCCGG
BTV-9/2/eup638R	CTGGTCCACATGGTAGATC
BTV-9/2/eup864R	CATCCGATAGACCGCCAC
BTV-9/2/eupFpp864F	GTGGCGGTCTATCGGATG
BTV-9/2/883R	GCGACTCCGCGCACATGTT
BTV-9/2/565F	GCGGATCCAGCAATAAAGCG
BTV-9/2/p491F	GGSAATATATTTCTRATGG
BTV-9 AUS-F	TGGAATGGATGTTATCGAC
BTV-9 AUS-R	GCATTCCGTTGCAATTAACG

Table 2-2 BTV-9 segment-2 (Seg-2) sequencing primers

Primer designation (aa)	Primer sequence (5'- 3')
BTV-16/2/Fpp151R	ATCGTATGATATGACGTGG
BTV-16/2/p151F	CCACGTCATATCATACGAT
BTV-16/2/p393F	GACTGCAAGCTCGGTGACG
BTV-16/2/p745R	GCGAGTCCGACACCTCAAG
BTV-16/2/Fpp745F	CTTGAGGTGTCGGACTCGC
BTV-16/2/p716R	CCTGCACTCATCGACTGAGC
BTV-16euro/2/TF	GTTAAAAACGTTAGCCTAGAG
BTV-16euro/2/TR	GTAAGTGTAAACGYGCCCAAT

Table 2-3 BTV-16 segment-2 (Seg-2) sequencing primers

Primer designation (aa)	Primer sequence (5'- 3')
BT-4/6/159F	TCAGGAACAATACTCAATGCT
BT-4/6/406R	GATTCATTCCGATGATGGTG
BT-4/6/FP159R	AGCATTGAGTATTGTTCCTGA
BT4/6/FP179R	CCATTTTCGAGTCCTCTGC
BT-4/6/FP406F	CACCATCATCGGAATGAATC
BT4/6/FP455F	TACGTTAACGGAGGCGTAT
S6BTV4F2	GTTAAAAAGTATTCTCCTACTC
S6BTV4R	GTAAGTGTAAGCTTCTCCCT
S6BTV4F1	GTTAAAAAGTGTTCTCCTCCTC
S6BTV4R	GTAAGTGTAAGCTTCTCCCT

Table 2-4 BTV-4 segment-6(Seg-6) sequencing primers

Primer designation (aa)	Primer sequence (5'- 3')
BT9EU/6/131F	GTCCAGGTGAGCGTGGGATT
BT9EU/6/119F	GAGTCCAGGTGAGCGTGGG
BT9EU/6/FP125R	CCCACGCTCACCTGGACTC
BT9EU/6/FP178R	ATATATCTCCTCTAATTCCCG
BT9EU/6/380R	GTCATCCGAATCCCATGG
BT9EU/6/506R	TCATCGTATACAATTCTTTGT
BT9EU/6/461R	CCTTATCATACGTCTAGCATGC
BT9EU/6/FP383F	CGTGTCGCATTTCCACGCC
BT9EU/6/FP463F	GCATGCTAGACGTATGATAAG

Table 2-5 BTV-9 segment-6(Seg-6) sequencing primers

Primer designation (aa)	Primer sequence (5'- 3')
BT16/6/73F	ACTCAATCATGACCGGGGA
BT16/6/FP126R	CTGCTCTTCTTCAATCTCCTT
BT16/6/426R	CCAATGGGCCGCCAAATCCTC
BT16/6/431R	GCKCCYAAAGCGTGCCAATG
BT16/6/FP426F	GAGGATTTGGCGGCCCATTGG
BT16/6/FP431F	CATTGGCACGCTTTRGGMGC

Table 2-6 BTV-16 segment-6(Seg-6) sequencing primers

2.5.2 : Sequence assembly and analysis

The raw sequence data obtained from the sequencer was analysed using CEQ8000 software. The SeqMan (DNAstar) sequence analysis package was used for the assembly of raw SCF files into 'contigs', allowing a consensus sequence to be obtained. Identification of the sequences obtained, was carried out using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5.3 : Sequencing reactions for amplified clones

10 µl volumes were made of the following

a) 1.88 µl buffer (supplied in the kit)

b) 0.25 µl reaction mix

c) Primer (either forward or reverse) to a final concentration of 3.2pmol

d) Template (5-20ng) and water to a final concentration of 10 µl.

These reactions were incubated for 25 cycles as follows: 96 °C forl min, 96 °C for 10 seconds, 55 °C for 5 seconds, 60°C for 4 minutes: then held at 4 °C. This was followed by the addition of 2.5µl of 125 mM EDTA and 30.0µl of 100% ethanol to each 10 µl reaction. The plate was sealed, mixed by vortexing for 15 sec, and then left in the dark at room temperature for at least 15 min to precipitate the extension products. The plate was centrifuged at (1400 to 2000 × g: 45 min or 2000 to 3000 × g: 30 min) and the supernatant was discarded as follows: without disturbing the precipitates, the adhesive tape was removed and the reaction plate inverted onto a paper towel folded to the size of the plate. The inverted reaction plate and paper towel are placed into the centrifuge and spun up to 185 × g. for one minute. This is followed by a 70% ethanol wash (30 µl per pellet). The wells were sealed and vortexed for 15 sec to mix. The reaction plate was placed in the centrifuge and spun for 15 min. at 1650 × g and the supernatant discarded as described above. The wells are dried using a Speed-Vac for 15 min. If samples were loaded immediately onto ABI- sequencer 3730 (Applied Biosystems) commonly known as Bubbles, 20 µl Hi-Di Formamide is added and the pellets are allowed to resuspend.

2.6 : Polymerase Chain Reaction (PCR)

2.6.1 : PCR amplification of cDNAs

Amplification of cDNAs was performed as described by Maan et al., (2007), using Primer '5-15-1' (5'-GAGGGATCCAGTTTAGAATCCTCAGAGGTC-3' - containing a Bam HI restriction site [underlined] – to aid cloning) and the Triple Master PCR system (Eppendorf). Reactions were carried out in a 10µl reaction mixture, containing 2µl (400ng) of cDNA product, 1µl 10x PCR buffer (tuning/high fidelity buffer with Mg2+), 8.0µl nuclease free water, 0.2µl dNTPs (10mM each stock), 0.2µl of 100uM 5-15-1 primer, and 0.5 U of Triple Master enzyme mix. Amplification was carried out after denaturation at 94 °C for 2 min, followed by 30 successive cycles of denaturation for 15 sec at 95 °C, annealing for 30 sec at 62 °C, extension for 4.30 min at 68 °C and a final extension step for 10 min at 68 °C.

2.6.2 : KOD polymerase PCR

KOD is a thermostable DNA polymerase derived from the bacterium Thermococcus kodakaraensis with an extremely low mutation frequency of 0.0035 (compared to 0.013 for Taq, 0.0039 for Pfu) (Takagi et al., 1997). KOD Hot Start reactions contained 5 µl of 10× PCR buffer, 3 µl 25 mM $MgSO_4$, 5 µl dNTPs (2 mM each), 15 pmols of each primer, 1 unit of KOD Hot Start polymerase and template/nuclease free water, to a final volume of 50 µl. Plasmid template (10 ng) or 2 µl of RT product was used in each reaction. Amplification conditions were as follows:

Step	Temperature (°C)	Time	Cycles
Polymerase activation	95	2 min	1
Denaturation	95	20 sec	35
Annealing	55-60[a]	10 sec	
Extension	70	20 sec per kb	
Final elongation	70	10 min	1

[a] the annealing temperature varied according to the melting temperature of the primers being used for amplification. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

Table 2-7 The amplification conditions thermal cycling used for KOD hotstart polymerase

2.6.3 : Real-time RT-PCR

Real-time RT-PCR was based on the method described by Shaw and others (2007). The superscript III/Platinum *Taq* one-step RT-PCR kit (Invitrogen) was used for all the real-time RT-PCR assays. A reaction mixture of 19 µl per sample was prepared containing the following components: 12·5 µl 2x reaction buffer mix (kit), 20 pmol of each primer (BTVuni 291-113F, BTVuni 381-357R, BTVrsa 291- 311F and BTVrsa 387-357R), 2·5 pmol of each probe (RSA-BTV 341-320 and BTV 346-323), 0·5 µl magnesium sulphate (kit), 0·5 µl ROX reference dye (kit) and 0·5 µl of the Superscript III/Platinum *Taq* enzyme mix (kit). The reaction mixture was added to the wells of reaction plates (Stratagene). Denatured RNA (6 µl) was added to the reaction mixture and the reaction capped with optical caps (Stratagene). Primers and probe sequences are described by (Shaw et al., 2007).

Amplification was carried out using Mx3005P (Stratagene) under the following conditions: 55°C for 30 minutes, one cycle (reverse transcription), 95°C for 10 minutes, one cycle (denaturation of the Superscript III and activation of the Platinum *Taq* DNA polymerase), and 50 cycles of 95°C for 15 seconds and 60°C for one minute.

Fluorescence was detected at the end of the 60°C annealing/extension step. Cycle threshold (Ct) values for each sample were determined from the point at which the fluorescence breached a threshold fluorescence line. A cycle threshold of less than 35 was considered positive for BTV RNA.

2.6.4 : Screening E. coli cultures for plasmid inserts using PCR

A few colonies from each transformation were picked using a pipette tip and seeded into 200µl LB broth containing 50 µg/ml ampicillin and incubated at 37 °C overnight, with shaking at 200 rpm. Two microlitres of each overnight liquid culture were added into a PCR mixture containing primers designed from the pGEX, pSC11 and pCI-neo vector. This is to identify colonies that contain the recombinant vectors with inserts of the right sizes. The PCRs were carried out in a PCR mix volume of 50 µl, containing: 5µl 10x PCR buffer, 5µl dNTP (10 mM stock), 1.5 µl forward primer (working concentration 10 µM) and 1.5 µl reverse primer (working concentration 10 µM), 1µl of DNA template (colony in liquid culture), 3µl of MgSO4, 1 µl of KOD Hot Start DNA Polymerase and 32 µl of water. The thermal cycling parameters were as listed in section (2.6.2: KOD polymerase PCR). Amplicons were analysed by agarose gel electrophoresis and purified on Qiagen column.

Target plasmid	PCR primers
pGEX4T2	pGEX-5' (GGGCTGGCAAGCCACGTTTGGTG) pGEX-3' (CCGGGAGCTGCATGTGTCAGAGG)
for pSC11	Generic pSC11-5' (TAATTTATTGCACGGTAAGG) longer pSC11-5' primer designed TAATTTATTGCACGGTAAGGAAGTAGAATCATAAAG Generic pSC11-3' (TKr) (GAAATGTCCCATCGAGTG) longer pSC11-3' designed GAAATGTCCCATCGAGTGCGGCTACTATAACTA
pCI-neo	pCI-neo-5' (GGTCTTACTGACATCCACTTTGCCTTTC) pCI-neo -3' (TAAAGCAATAGCATCACAAATTTCACA)

Table 2-8 Primers used for colony screening

2.6.5 : One-step RT-PCR

One-step RT-PCR was used for RT-PCR analysis of the viral RNA extracted from cell sheets (CEF) infected with rMVA-BTV, as described in section (2.3.2). The Roche Transcriptor one-step RT-PCR kit was used for reverse transcription and PCR amplification in a single reaction, according to the manufacturer's instructions. The RT-PCR reactions contained 10 µl of 5× mastermix (Qiagen), 0.4µM of each primer, 1 µl of

Transcriptor enzyme mix, 100ng of RNA Template and water to a final volume of 50 µl. Amplification conditions were as follows

Step	Temperature (°C)	Time	Cycles
Reverse transcription	50	5 min	1
Initial denaturation	94	5 min	1
PCR Profile			
Denaturation	94	10 sec	35
Annealing	Depends on melting temperature of the primers used [a]	30 sec	
Extension	68	60 sec per kb	
Final elongation	68	5 min	

[a] the annealing temperature varied according to the melting temperature of the primers being used for amplification (oligonucleotide primers used to amplify each BTV gene).

The PCR products were visualized on a 1% agarose gel stained with ethidium bromide

Table 2-9 The amplification conditions /thermal cycling used for Detection of DNA amplicons generated from RNA transcripts

2.7 : Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) was used for analyses of PCR products and to separate DNA fragments according to their molecular weight/size, allowing specific DNA fragments to be recovered. Agarose gels were stained with ethidium bromide (0.5µg/ml) and run at 120V. For DNA fragments greater than 200 bp a 1 % gel was used with Hyperladder I (Bioline) or DNA Molecular Weight Marker III (Roche) as a DNA size markers.

2.8 : Cloning

BTV-8 (NET2006/04) Seg-2, Seg-6, Seg-7 and BTV-6 (NET2006/04) Seg-7 cDNA and its corresponding sequence were obtained from Drs. Andrew Shaw and Sushila Maan (IAH Pirbright). pBRT7 BTV-8 Seg-2, pBRT7 BTV-8 Seg-6 and (pBRT7 BTV-8 Seg-7 pBRT7 and BTV-6 Seg-7) which encode VP2, VP5 and VP7 respectively, were generously provided by Dr Andrew Shaw.

2.8.1 : cDNA cloning into pGEX-4T2 vector

2.8.1.1 : Cloning of BTV-8 segment 2 into modified pGEX4T2 vector

a) Cloning of BTV-8 Seg-2 as three separate but overlapping fragments

Because of the large size of Seg-2 (~2950bp) and the in-soluble nature of the protein in bacterial expression systems, we have chosen to clone the open reading frame into pGEX vector, as three separate but overlapping RT-PCR products. The overlap consisted of approximately 375 bp (encoding 125 amino acids). A schematic of the cloning strategy is shown in AppendixIII.

b) Cloning of BTV-8 Seg-2 as a complete segment

BTV-8 Seg-2 was also cloned as a complete open reading frame into modified pGEX4T2 as a RT-PCR product using the forward primers (BTV8_VP2_Frag1_For1 and BTV8_VP2_Frag1_For2) and the reverse primer BTV8_VP2_Frag3_Rev. The primers used for cloning the complete Seg-2 are shown in table 2.4.

2.8.1.2 : Cloning of BTV-8 segment 6 and BTV-6 segment 7 into modified pGEX4T2 vector

BTV-8 Seg-6 is 1638 bp, while BTV-6 Seg-7 is 1156bp. Because of the problems encountered and failure of expression VP5 as a complete protein in Prokaryotic expression system; approximately 1300bp (starting from bp 261 as shown in appendix IV and V) of BTV-8 segment 6 was cloned into modified pGEX4T2 as a RT-PCR product. The open reading frame of BTV-6 segment 7 was cloned into modified pGEX4T2 as RT-PCR products.

Primers (shown in table 2.6), were designed to introduce ECORI and NOTI restriction sites respectively into the 5' and 3'of the PCR products of the gene of interest. This allows digestion and cloning into ECORI and NOTI digested pGEX4T2 vector.

2.8.2 : cDNA cloning into MVA transfer plasmid (pSC11 vector)

2.8.2.1 : Cloning of BTV-8 Seg-2, BTV-8 Seg-6 and BTV-6 Seg-7 into pSC11 vector

To generate MVA transfer plasmid, (pSC11-Seg-2, pSC11-Seg-6 and pSC11-Seg-7). The open reading frames (ORF) of BTV-8 segment 2, BTV-8 segment 6 and BTV-6 segment 7 were amplified by PCR, using primers (shown in table 2.7) that introduce SmaI restriction site into the 5' and 3'of the PCR products of the gene of interest. This allows digestion and cloning into SmaI digested pSC11

2.8.3 : cDNA cloning into pCI-neo vector to generate DNA vaccines, (pCI-neo-Seg-2, pCI-neo-Seg-6 and pCI-neo-Seg-7)

The Open Reading Frames (ORF) of BTV-8 segment 2, BTV-8 segment 6 and BTV-8 segment 7 were amplified by PCR, using primers (shown in table 2.8) that introduce XbaI and NOTI restriction site into the 5' and 3'of the PCR products of the gene of interest. This allows digestion and cloning into XbaI and NOTI digested pCI-neo vector.

2.8.4 : Vectors

2.8.4.1 : pGEX4T2

pGEX4T2 was used for expression purposes (in which the thrombin cleavage site was replaced by a precision-protease cleavage-site, previously prepared and generously provided by Dr. Houssam Attoui -IAH). pGEX4T2 allows expression of an amino terminus GST fusion protein with 6xHis tag at the carboxyl terminus. The GST tag can be used for purification of the fusion protein using immobilised glutathione attached to sepharose beads. The 6xHis tag can also be used for purification of the recombinant protein using nickel-affinity-columns. The target protein can then be cleaved away from GST using the precision-protease-site. NNNNNNCTGGAAGTTCTGTTCCAGGGTCCC. pGEX4T2 vector has the EcoRI restriction site (GAATTC) and NotI restriction site (GCGGCCGC) in its sequence.

The full sequence of the modified pGEX4T2 is included in annex 2. The sequence below shows the region encoding the precision-protease site (in bold and undelined) and the EcoRI and NotI cloning sites (red bold). The sequence of the modified pGEX4T2, encoding the 6xHis tag (green italic bold) is followed by two successive stop codons (highlighted in red).

.....**CTGGAAGTTCTGTTCCAGGGTCCC**GAATTCCCGGGTCGACTCGAG
CGGCCG*CATCATCACCATCACCAT*TAGTGA...

2.8.4.2 : pSC11

To achieve expression of foreign genes in MVA, the gene of interest was inserted into pSC11 at the SmaI restriction site (**CCCGGG**) immediately downstream of the constitutive vaccinia promoter p7.5. The lacZ gene is built into the plasmid adjacent to the insertion site and is controlled by the vaccinia late promoter p11. This expression cassette is flanked by vaccinia thymidine kinase DNA sequences which allow its integration into the MVA genome at the TK locus. Recombinant viruses will therefore

express the lacZ gene, allowing them to be selected as blue plaques after staining with X-gal. The full sequence of the Psc11 is included in annex 2

2.8.4.3 : pCI-neo

To generate DNA vaccines, the gene of interest was inserted in pCI-neo mammalian expression vector using the Xbal (TCTAGA) and NotI (GCGGCCGC) restriction sites, downstream from the cytomegalovirus (CMV) promoter.

2.8.5 : Primer design

2.8.5.1 : Primers used for cloning in pGEX4T2

2.8.5.1.1 : BTV-8 Seg-2

Several pairs of primers (shown in table 2.4), were designed for the cloning of the overlapping fragments into expression vectors.

a) **Fragment 1**: is 1209 bp-long. The nucleotide sequence of this fragment starts at the ATG initiation codon (the A of the ATG being designated position 1) until position 1209.
b) **Fragment 2**: is 1215 bp-long. The nucleotide sequence of this fragment starts at position 835 until position 2049.
c) **Fragment 3**: is 1209 bp-long. The nucleotide sequence of this fragment starts at position 1675 until position 2883.

These fragments were designed for BTV-8 NET2006/04 (Segment-2) and accordingly using any other serotype and/or strain would require different primer designs in accordance to the position of the restriction sites, which may vary.

Fragment 1 contained an EcoRI restriction site within its sequence at position 147-152. Because cloning into the modified pGEX4T2 involves EcoRI and NotI sites, it was not possible to simply use a primer containing an EcoRI site, and restriction digestion of the cDNA product. An alternative approach was therefore used to generate a cleaved EcoRI site in the cDNA products as described below.
Open Reading Frame (ORF) fragments 2 and 3 were amplified by PCR using
(BTV8_VP2_Frag2_For and BTV8_VP2_Frag2_Rev) for fragment 2 amplification
(BTV8_VP2_Frag3_For and BTV8_VP2_Frag3_Rev) for fragment 3 amplification (as shown in table 2.10)

These primers contain the restriction enzyme sites for EcoRI and NotI, allowing conventional digestion and cloning in pGEX-4T2, and expression of the target protein as a fusion protein with GST. The primer sequences used to amplify fragments 2 and 3 for cloning into pGEX4T2 are shown below in table 2.10:

d) BTV-8 Seg-2 complete segment

BTV-8 Seg-2 was cloned into a modified pGEX-4T2 vector. The primers used were BTV8_VP2_Frag1_For1, BTV8_VP2_Frag1_For2 and BTV8_VP2_Frag3_Rev shown in Table 2.4. To avoid digestion of the PCR product of fragment 1 by EcoRI, an alternative cloning strategy was used. Two PCR products were amplified

Product one was amplified using the forward primer BTV8_VP2_Frag1_For1 primer and the reverse primer BTV8_VP2_Frag3_Rev.

Product two was amplified using the forward primer BTV8_VP2_Frag1_For2 primer and the reverse primer BTV8_VP2_Frag3_Rev. All primers are shown in table 2.10

Primer	Primer sequence : 5'→3' For cloning into PGEX4T2	Fragment amplified
BTV8_VP2_Frag1_For1	AATTCATGGAGGAGCTAGCGATTCCG	Fragment 1
BTV8_VP2_Frag1_For2	CATGGAGGAGCTAGCGATTCCG	
BTV8_VP2_Frag1_Rev	ATGATGGCGGCCGCGTTCTCTATTCCTATCGTAAGGTAC	
BTV8_VP2_Frag2_For	gcagctGAATTCTTAGATCTGTGTAGGTTGTTGTC	Fragment 2
BTV8_VP2_Frag2_Rev	ATGATGGCGGCCGCGTTCAAACAAATAGTCAAATACGAA	
BTV8_VP2_Frag3_For	gcagctGAATTCTTTGACCTGCAGCGATATTGTC	Fragment 3
BTV8_VP2_Frag3_Rev	ATGATGGCGGCCGCGTACATTGAGCAGCTTAGTTAAC	

Table 2-10 Primers designed for amplification of the fragments of Seg-2 to be cloned into pGEX4T2. The underlined parts of the primers are those from the specific sequence of Seg- 2. Bold red sequences are those of the restriction enzymes EcoRI and NotI. NB: in primers designated Frag1_For1 and Frag1_For2, there is not full EcoRI site. As explained in the text, I have used these primers in combination with the primers designated Frag1_Rev and the resulting PCR products were mixed together in order to create an EcoRI site as it would have existed following a restriction digestion.

To avoid digestion of the PCR product of fragment 1 or complete Seg-2 by EcoR1, an alternative cloning strategy was used.

Two PCR products were amplified with the same reverse-primer

BTV8_VP2_Frag1_Rev(ATGATGCGCCGGCGcTTCTCTATTCCTATCGTAAG GTAC) which contains a NotI restriction site, but using two distinct forward-primers were used (designated BTV8_VP2_Frag1_For1[AATTCATGGAGGAGCTAGCGATTCCG]& BTV8_VP2_Frag1_For2 [CATGGAGGAGCTAGCGATTCCG]) for amplification of Fragment 1.

In the same manner to avoid digestion of the PCR product of complete Seg-2 by EcoR1, the same strategy described above was used where two PCR products were amplified with the same reverse-primer

BTV8_VP2_Frag3_Rev(ATGATGGCGGCCGCGTACATTGAGCAGCTTAGTT AAC), but using the two Frgment 1 forward-primers BTV8_VP2_Frag1_For1[AATTCATGGAGGAGCTAGCGATTCCG]& BTV8_VP2_Frag1_For2 [CATGGAGGAGCTAGCGATTCCG])

All primers are shown in table 2.10

A schematic of PCR product preperation of Fragmen1 and complete Seg-2 used for cloning in pGEX vector is shown in table 2.11 bellow

The use of these reverse primer plus forward primer 1 generated the following products:

AATTCATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGCCGGCGCATCAT
TTAAGTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCCGCGTAGTA

The use of reverse plus forward 2 generate these product:

CATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGCCGGCGCATCAT
GTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCCGCGTAGTA

Digesting both products with NOTI (GCGGCCGC) gives these products

AATTCATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGCCGGCGCATCAT
TTAAGTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCCGCGTAGTA

And

CATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGCCGGCGCATCAT
GTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCCGCGTAGTA

NNNNNN represents the specific sequence of the fragment amplified by the primers.

Both products were purified using Qiagen columns

AATTCATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGC
TTAAGTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCC

And

CATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGC
GTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCC

The two products are then mixed together and heated to 100 °C for 5 minutes then left to cool and re-anneal, generating a mixture of 4 products. These include the two initial amplicons, plus two others as shown below.

The two original products are:

AATTCATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGC
TTAAGTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCC

And

CATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGC
GTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCC

The two new products generated by the mixing, boiling and re-annealing are:

ATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGC
TTAAGTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCC

And

AATTCATGGAGGAGCTAGCGATTCCGNNNNGTACCTTACGATAGGAATAGAGAACGC
GTACCTCCTCGATCGCTAAGGCNNNNCATGGAATGCTATCCTTATCTCTTGGCGGCC

The last product contains half of an EcoR1 restriction site, generated by the terminal overlap of the two different PCR products and it can be inserted into a pGEX4T2 that was previously digested with both EcoRI and NotI.

Table 2-11 PCR product preparation of Fragmen1 and complete Seg-2 used for cloning in pGEX vector

2.8.5.1.2 : BTV-8 segment 6 and BTV-6 segment 7

Primers were designed for the amplification (by PCR) and cloning of the ORFS from BTV-8 NET2006/04 (Segment-6) and BTV-6 NET2006/04 (Segment-7) (table 2.12). These primers contain restriction enzyme sites for EcoRI and NotI, allowing conventional digestion and cloning into pGEX-4T2, and expression of the target protein as a fusion protein with GST.

Primer	Primer sequence :5' → 3' For cloning into PGEX4T2
BTV8_Seg6_VP5_ For truncated	gcagctGAATTCATGAATGGGGTTGCGAAGCAGGAG
BTV8_Seg6_VP5_ For complete	gcagctGAATTCATG**GGGAAAATCATAAAGTCCC**
BTV8_Seg6_VP5_ Rev	ATGATGGCGGCCGCgGGCATTTCTTAAGAAGAGTGG
BTV6_Seg7_VP7_ For	GcagctGAATTCATGGACACTATCGCAGCAAGAGCG
BTV6_Seg7_VP7_ Rev	TGATGGCGGCCGCgTGCGTAAGCGGCGCGAGCAAT

Table 2-12 Primers designed for amplification of the ORFs from segments 6 and 7 to be cloned into pGEX4T2.

The underlined parts of the primers are those from the specific sequence of Seg- 6 and Seg-7. Bold red sequences are those of the restriction enzymes EcoRI and NotI.

2.8.5.2 : Primers used for cloning into pSC11

To generate MVA transfer plasmid, (pSC11- BTV-8- Seg 2, pSC11- BTV-8 Seg-6 and pSC11- BTV-6-Seg-7). Briefly, the Open Reading Frames (ORF) for BTV-8 NET2006/04 (Segment-2 and Segment-6) and BTV-6 NET2006/04 (Segment-7), were amplified by PCR using gene specific primers (Table 2.7) containing a SmaI restriction site and cloned into the SmaI site of the standard Vaccinia transfer vector pSC-11, generating plasmids pSC-11 BTV-8 Seg- 2; pSC-11 BTV-8 Seg- 6; and pSC-11 BTV-6 Seg-7 (respectively) , so that these genes could be expressed under the control of the Vaccinia virus P7.5 promoter.

The primer sequences used to amplify BTV-8 (Seg-2), BTV-8 (Seg-6) and BTV-6 (Seg-7) for cloning into pSC11 are shown in Table 2.13:

Primer	Primer sequence : 5' ⟶ 3' For cloning into pSC11
f/BTV8_VP2_vac	TTTTCCCGGGACCATGGAGGAGCTAGCGATTCCGAT
r/BTV8_VP2_vac	TTTTCCCGGGCTATACATTGAGCAGCTTAG
f/BTV8_VP5_vac	TTTTCCCGGGACCATGGGGAAAATCATAAAGTCCCTAAGC
f/BTV8_VP5_vac	TTTTCCCGGGCTAGGCATTTCTTAAGAAGAGTGG
f/BTV6_VP7_vac	CCCGGGACCATGGACACTATCGCAGCAAGAGCGCTCAC
f/BTV6_VP7_vac	CCCGGGCTATGCGTAAGCGGCGCGAGCAATCGCACGTGTAAG

Table 2-13 Primers designed for amplification of the ORFS from Seg-2, Seg-5 and Seg-7 to be cloned into pSC11.
The underlined parts of the primers are those from the specific sequence of Seg- 2, Seg-6 and Seg-7. Bold red sequences are those of the restriction enzyme SmaI.

2.8.5.3 : Primers used for cloning in pCI-neo vector: to generate DNA vaccines

The Open Reading Frames (ORF) from BTV-8 NET2006/04 (Segment-2, Segment-6 and Segment-7), were amplified by PCR using primers that introduce XbaI and NotI restriction site into the 5' and 3'of the PCR products (table 2.14). This allows digestion and cloning into XbaI and NotI digested pCI-neo vector to generate (CI-neo BTV-8 Seg 2, pCI-neo BTV-8 Seg 6 and pCI-neo BTV-8 Seg 7.

Primer	Primer sequence : 5'⟶3' For cloning into pCI-neo
f/BTV8_Seg2_DNA	GCATTTTCTAGAATGGAGGAGCTAGCGATTCCGATTTAT
r/BTV8_Seg2_DNA	CGTAAAGCGGCCGCGCTATACATTGAGCAGCTTAGTTAACAT
f/BTV8_Seg6_DNA	GCATTTTCTAGAATGGGGAAAATCATAAAGTCC
r/BTV8_Seg6_DNA	AAATGCGCGGCCGCGTCAGGCATTTCTTAAGAAGAG
f/BTV8_Seg7_DNA	GCATTTTCTAGAATGGACACTATCGCTGCAAGAGCA
r/BTV8_Seg7_DNA	CGTAAAGCGGCCGCGCTAAGAGACGTTTGAATGGGTTAC

Table 2-14 Primers designed for amplification of the ORFs from segments 2, 6 and 7 to be cloned into pCI-neo vector
The underlined parts of the primers are those from the specific sequence of Seg- 2, Seg-6 and Seg-7. Bold red sequences are those of the restriction enzyme XbaI and NotI.

2.8.5.4 : Primers used for verifying the complete VP2 sequences

To confirm the complete sequence of VP2 used for Bacterial expression, rMVA expressing VP2 and BTV-8 Seg-2 DNA vaccination. Six internal primers were designed to cover the entire sequence of VP2. The primer sequences used are shown in table 2.15:

Primer	Primer sequence : 5' ⟶ 3'
BTV8S2-650/F	CGAATTAGGTCGTAATCATCAAATTCA
BTV8S2-2300/R	GGTATAATTCGGATATCGTGCGCA
BTV8S2-1200/F	TAGAGAAAGTGATAAGTATATTTATAG
BTV8S2-1800/R	ACGCCTTGAAACAATCCCTGC
BTV8S2-650/R	ATCATAGTTCCATTAAAGGAATC
BTV8S2-2300/F	TGCGCACGATATCCGAATTATACC

Table 2-15 Primers designed for confirmation of the complete sequence of BTV-8 Seg-2 used for bacterial expression, MVA expression and DNA vaccine preparation.

2.8.6 : Cloning reactions

2.8.6.1 : Amplification and processing of the products to be inserted in vectors

a) PCR amplification for cloning into pGEX4T2 vector

PCR mixtures with final volumes of 100 µl were prepared, containing: 10µl of 10X PCR buffer, 5µl forward primer (stock concentration 10 µM), 5µl reverse primer (stock concentration 10 µM), 2µl dNTP (10 mM stock concentration), 0.5 units of Taq polymerase, 1µl of DNA template (previously amplified by the FLAC) and 73µl of nuclease free water. The thermal cycling parameters were: 94 °C for 4 minute x 1 cycle, followed by 40 successive cycles of 94 °C for 50 seconds, 55 °C for 1 minute and 68°C for 2 minutes. The cycling was terminated by a final extension step at 68°C for 10 min. The products were analysed by agarose gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

b) 'Polishing' the purified PCR products

Taq polymerase has the property of terminal deoxynucleotidyl transferase, allowing it to transfer particularly a deoxy-adenosine residue onto each of the 3' ends of the PCR products. A 'polishing-step' is therefore necessary to remove the A-residue from the 3' end, using a proof reading enzyme such as pfu or pfx polymerase. The products used

to generate fragment 1 (by mixing, boiling and re-annealing as described above) need to be polished to remove the A-residue, creating the partial EcoRI site, in order to make them 'clonable' into pGEX4T2.

The pfx polymerase was used in the following mixture. 5 µl of pfx 10x buffer supplied, 1µl of MgSO4 (final concentration of 0.2 mM), 1µl of pfx polymerase, 2.5µl of dNTP (final concentration of 0.5mM each), 40 µl (10 ng) of DNA (template) and 0.5µl of nuclease free water, to a final volume of 50µl. The reactions were incubated at 68°C for 30 minutes, followed by purification of the 'polished' product using Qiagen column.

c) PCR amplification for cloning into pSC11 and pCI-neo vector

KOD polymerase PCR was used as described in (2.6.2). For cloning into pSC11, primers (table 2.7), were used to introduce the SmaI restriction site into the 5' and 3'of the PCR products. For the purposes of cloning into pCI-neo, primers (table 2.8), were used to introduce the XbaI and NotI restriction sites into the 5' and 3'of the PCR products respectively.

2.8.6.2: Restriction digestion reaction

The PCR products prepared for the ORF of Seg-2, using the primers defined in (Table 2.10), were digested using the appropriate restriction enzymes (EcoRI and NotI). For fragment 1 PCR products, only NotI was used. In the case of the PCR products of fragments 2 fragment 3, BTV-8 Seg-6 and BTV-6 Seg-7, these were double digested with EcoRI and NotI.

The PCR products from the ORF of Seg-2, Seg-5 and Seg-7, prepared using the primers defined in Table 3, were digested using SmaI. The PCR products prepared from the ORFs of segments 2, 6 and 7 using the primers defined in Table 4, were digested using XbaI and NotI restriction enzyme.

Each restriction digestion reaction contained 3ul of appropriate buffer, 25µl of the PCR products (cDNA) and 1ul of the appropriate restriction enzyme (for PCR products digested with one restriction enzyme or 1 ul of each restriction enzyme for the double digest). In each case the relevant vectors were digested using the same restriction enzymes .The reaction mix was made of 2µl of buffer, 1µg of vector (pGEX4T2, pSC11 or pCI-neo vector), 1ul of the appropriate restriction enzyme (for vectors digested with one restriction enzyme or 1 ul of each restriction enzyme for the double digestion of vectors). A final volume of 20 µl was made up with nuclease free water.

The reactions were then incubated at 37°C overnight. The enzymes used were inactivated by incubation at 70°C for five minutes. Agarose gel electrophoresis (AGE) was used to analyse the digested constructs, which were recovered and purified in accordance with the manufactures instructions (Qiagen) for gel purification.

2.8.6.2 : Dephosphorylation

The Digested pSC11 vector was gel purified before de-phosphorylation for 15 minutes at 37 °C using SAP (Promega); according to the manufacturer's instructions. This was followed by an inactivation of the enzyme at 65°C for 20 minutes.

2.8.6.3 : Ligation reaction

The amount of DNA needed in the ligation reaction, was estimated by visual comparison of the PCR products to a quantitative DNA ladder after agarose gel electrophoresis (AGE). A standard ligation reaction consisted of 20 ng of purified digested plasmid vector (and dephosphorylated for pSC11 vector). The insert was digested using the same restriction enzyme, then mixed with the vector at molar ratios of vector to insert, of 1:1, 1:3 or 3:1, in reactions containing 2 µl 5x ligation buffer (Promega), 1 U of T4 DNA ligase and nuclease free water to a final volume of 10 µl. Ligations were carried out at 16 °C overnight before transfection into appropriate chemically competent *E. coli* cells.

2.9 : Transformation of chemically competent cells with plasmids

Several bacterial strains were used in these studies including: XL1-Blue competent cells (Stratagene); BL-21 competent cells (Stratagene), C41 (DE3) and C43 (DE3).

The XL1-Blue bacterial strain was used for cloning recombinant pGEX4T2, pSC11 and pCI-neo vectors. BL-21 was used for transforming minipreps of the clones with the correct junctions and correctly oriented inserts, in preparation for bacterial protein expression. C41 (DE3) and C43 (DE3) were used in an attempt to express VP5, using identical transformation methods for both strains.

Competent XL1-Blue bacteria were thawed on ice and 40 µl aliquots were prepared in pre-chilled Eppendorf tubes. For each transformation, 0.7 µl of β-mercaptoethanol was added (1.42 M, provided with the competent bacteria) and the mixture was incubated for 10 minutes on ice. 2.5µl of ligation-reaction was added to each transformation, and then mixed gently before incubating on ice for 30 minutes. The XL1-Blue bacteria

were heat shocked for 45 seconds at 42 °C and immediately placed on ice for two minutes.

450 µl of L-B Broth (see Appendix XIII a) was added and the tubes were shaken at 200 rpm at 37 °C for 1 hour before 100µl was spread on LB agar plates containing the 50 µg/ml ampicillin. After spreading the transformed cells, the plates were inverted and incubated at 37 °C overnight. (The composition of the agar plates can be found in Appendix XIII b)

2.10 : Microcultures

2.10.1 : Bacterial minipreps

All buffers used were supplied in the kit and are listed in Appendix .Pelleted bacterial cells were re-suspended in 250µl Buffer P1 and transferred to a microcentrifuge tube. 250µl Buffer P2 was added, the tube was inverted 4–6 times to ensure thorough mixing. 350µl Buffer N3 was added and mixed thoroughly by inverting the tube 4–6 times. The mixture was centrifuged for 10 min at 16000 x *g*, forming a compact white pellet. The supernatant was recovered and applied to a QIAprep spin column, then centrifuged for 1 min at 12000 x *g* and the flow-through was discarded. The column was washed by adding 750µl of Buffer PE and centrifuging for 1 min at 12000 x *g*. The flow-through was discarded, and the column centrifuged for an additional 1 min at 16000 x g to remove residual wash buffer. The column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of water (preheated at 60°C) was applied to the centre of the QIA quick membrane and the column centrifuged for 1 min at 16000 x g. The DNA was used immediately for transformation or stored at -20°C and for vaccination with DNA vaccines.

2.10.2 : Bacterial midiprep

The Qiagen 'HiSpeed mididkit' was used according to the manufacturer's instructions to prepare large volumes of plasmid for DNA vaccines

2.11 Protein expression

2.11.1 : Protein expression in 100 ml medium

An overnight culture was prepared in 10 ml L-B medium in presence of 100 µg/ml ampicillin and incubated overnight at 37°C in a shaking incubator (220 rpm). The next day, 100 ml of fresh 2XYT culture medium (see Appendix VIII d) was inoculated with 1 ml of the overnight culture in presence of 100 µg/ml ampicillin. This was incubated at 37°C in the shaking incubator until reaching OD600 of 0.5-1.0. The culture was cooled

on ice for 15 minutes, followed by induction with 0.5 mM IPTG and continued incubation at 30°C for 4 hours. The culture was then kept at 4°C. The cells were harvested by centrifugation at 3500rpm in 50 ml centrifugation tubes for 25 minutes. The supernatant was removed and the pellet re-suspended in 7.5 ml of PBS containing EDTA-free, complete cocktail anti-protease (Roche) and 1 ml lysozyme (10 mg/ml). The mixture was incubated on ice for approximately 15 minutes. This was followed by sonication with a microtip in a Vibracell sonicator for a total of 5 minutes, at 20% output, using 2-second pulses and 4-second pauses. TritonX-100 (20%) is added to a final concentration of 1%, 10 µl of Benzonase (Novagen) to the homogenate and the mixture was incubated with gentle agitation at room temperature for 30 minutes. The homogenate was centrifuged at 3500 rpm (10°C) for 30 minutes and the supernatant (cell lysate) was separated from the pellet (the pellet as stored at -20°C). 300µl of Glutathione Sepharose beads (Amersham) was added to the cell lysate and the mixture was incubated for 30 minutes with gentle agitation, then introduced into a column (empty PD-10 column, Amersham) and flow-through collected. The unbound proteins were washed from the resin by adding 5 volumes of PBS to the pellet. The column outlet was closed and 300 µl of reduced glutathione was placed in the column which as rotated for another 30 minutes. The protein was recovered from the outlet and placed in sterile tubes. 20 µl of each fraction was analysed by 10% SDS-PAGE gel.

2.12 : Electrophoresis, staining of protein bands

For Preparation of 10% (separating) and 3% (stacking) SDS-PAGE gel for protein analysis and electrophoresis are shown in (AppendixVIII f). The 10% gel was poured into the electrophoresis apparatus followed by an overlay of running buffer, this was left to polymerise at room temperature for 30-45 minutes. When polymerisation as complete the overlay was poured out and the stacking gel (3%) was poured over the separating gel (10%) and a teflon comb is inserted. The gel was left to polymerise for 45 minutes before removal of the comb and sample loading.

20µl of purified proteins was mixed with an equal volume of TSTD solution (see AppendixVIII d for preparation), then heated at 96°C for 5-10 minutes. During this time the gel wells were thoroughly washed, to remove non-polymerised acrylamide solution. The denatured protein as loaded into the wells using the fine micropipette tip without overflowing to neighbouring wells (approximately 20-25 µl). 10 µl of dual colour protein molecular weight marker (BioRad) was loaded in one of the wells. The wells not loaded with protein were loaded with 10 µl of TSTD solution. The gel was placed in the

tank with 1x running buffer (see AppendixVIII g) and as run at 160V for approximately 1 hour, or until the blue dye-front comes out from the bottom of the gel. The gel was removed from glass plates and incubated in coomassie blue staining solution with gentle agitation for1-2 hour. For de-staining, the gel was placed in ultrapure water to remove blue background and bands could be visualized. The gel was then dried under the vacuum at 60°C for 1hr.

2.13 Western immuno-blot

In order to transfer proteins onto nitrocellulose membranes, Whatman filter paper was prepared and cut to the size of the gel. The sponge and filter paper were immersed in transfer buffer (see AppendixVII-i-1) prior to assembly. Starting with one layer of sponge material followed by two sheets of filter paper, the transfer membrane was placed on top of the filter paper. The gel was placed on top of the Nitrocellulose membrane (Bio- Rad). The transfer stack was completed by putting the two remaining sheets of filter paper on top of the gel. The stacked sandwich was placed vertically into a tank filled with transfer buffer. Transfer was performed at 160 mA for 90 minutes. The membrane was recovered and placed in a plastic pouch with the blocking buffer made of milk powder in PBS at a concentration of 5%. This was left at room temperature with gentle agitation for 30 minutes. The primary antibody (anti GST antibody) was diluted in blotting solution (1/750). The membrane was incubated with the diluted primary antibody in the plastic pouch and left overnight at room temperature, with gentle agitation. The next day, the membrane is washed three times (10 minutes each time) in Tris-Buffered Saline- Tween 20 (TBST) (see AppendixVIII-i-2).

The secondary antibody (monoclonal antirabbit immunoglobulins-peroxidase antibody produced in mouse) (Sigma) was diluted in Blocking solution II (see AppendixVIII-i-4). The membrane was placed in the diluted secondary antibody, in a plastic pouch and left at room temperature for 2 hours with gentle agitation. The membrane was washed three times (10 minute each) in TBST. The membrane was incubated with freshly prepared detection solution (see AppendixVIII-i-5) in the dark with gentle agitation for 30 minutes. The membrane was washed with water to stop the reaction, and dried in air.

2.13.1 : Preparation of western immunoblot strips for antibody detection

SDS-PAGE was performed at 160 mA for approximately 1 hour using a virus lysate, obtained from harvesting infected cells showing 100 CPE, then centrifuged at 3000 rpm for 10 min. 80 ul of the supernatant (virus lysate) was mixed with an equal volume of TSTD (Laemmli loading buffer) and incubated at 96°C for ten minutes. 80 µl was

loaded onto a large well using a fine micropipette tip. 10 µl of dual colour protein molecular weight markers (Bio-Rad) was loaded into a small well.

Proteins were transfered onto nitrocellulose membranes as described above (section 2.13).The membrane was recovered and cut into strips which were left to dry. These strips were blocked in (blocking solution I) containing skimmed milk at a 5% w/v concentration with gentle agitation for approximately 1hr. The primary antibody (present in serum obtained from the animal experiment) as diluted (1:50) in Blotto solution I containing skimmed milk at a 5% w/v concentration.

The membrane was incubated with the diluted primary antibody in a 1ml syringe and left at room temperature overnight with gentle agitation. The next day, the membrane was washed twice for 10 minutes in TBST. Secondary antibody Anti-Mouse IgG (Fc specific)–Peroxidase antibody produced in goat (Sigma) was diluted with Blocking solution II containing skimmed milk at a 5% w/v concentration. The membrane strips were incubated with the diluted secondary antibody in a 1ml syringe and left at room temperature for two hours with gentle agitation. The, membrane strips were washed twice for 10 minutes in TBST. The strips were incubated with freshly prepared detection solution in the dark, with gentle agitation for approximately 30 minutes, washed with water to stop the reaction and left to dry it in air.

2.14 : Purification of insoluble fraction from inclusion bodies

The pellet prepared (as described in section 2.14), was re-suspended in 2 ml Bugbuster reagent (Novagen). Lysozyme (from 10mg/ml stock) to a final concentration of 200µg/ml is added to the suspension, and the mixture is incubated with gentle agitation at room temperature for 5 minutes. 6 volumes of diluted (1: 10) Bugbuster reagent was added to the mixture and gently vortexed. This was followed by centrifugation of the mixture at 3.500 rpm for 15 minutes. The supernatant was removed and the pellet re-suspended in ½ original culture volume in diluted (1:10) Bugbuster reagent, the mixture is vortexed and centrifuged at 3500 rpm for 15 minutes. This step was repeated three times. The maximum amount of the supernatant was removed.

The tube containing the resulting pellet as weighed to obtain pellet weight and was kept on ice. 10X solubilisation buffer (CAPS 500 mM pH 11) was prepared, and 1X solubilisation buffer with N-lauroylsarcosine to final concentration of 0.3% (use 30% N-lauroylsarcosine (NLS) stock) and 1 mM DTT (use 1 M DTT stock) was prepared.

The pellet (containing inclusion bodies) was re-suspended in 1X solubilisation buffer containing NLS and DTT to obtain a final concentration of inclusion bodies of 10-20 mg/ml. The mixture was incubated with gentle agitation at room temperature for 15 minutes. The mixture was centrifuged at 2000 g for 10 minutes, and the supernatant was recovered carefully without touching the pellet (if there was any).and placed in sterile tubes and stored at -20°C. 20 µl of each fraction was analysed on a 10% SDS-PAGE gel.

2.15 : Protein preparation for animal inoculation

Purified protein (see sections 2.14) was removed from the -20°C freezer and left to thaw. One ml of the thawed protein was placed in a 15 ml falcon tube; 1 ml of PBS is added to the protein. The vivaspin protein concentrator (Vivascience) with a Molecular Weight Cut Off (MWCO) of 3KDa that allows protein concentration based on ultrafiltration and membrane adsorption techniques were used to concentrate fragmented VP2 proteins (Frag.1, Frag.2 and Frag.3) used for inoculation of the mice in the pilot animal experiment. The vivaspin concentrator was prepared by damping the column membrane with 500 µl of PBS, centrifuged at 2000 rpm for 10 minutes at 20°C. The diluted protein was transferred into the column and centrifuged at 2000 g, 15°C for 20 minutes. The flow through was placed in tubes for future use as blank. The Vivaspin concentrator has a concentrator membrane with a pore size two and a half times smaller than the molecular weight of the protein thus allowing recovery of the protein from the upper part of the device. The protein was pipetted and placed into screw capped tubes; the protein concentration was measured using a spectrometer in order to calculate the volume of protein required for the animal inoculation.

2.16 : Pilot animal vaccination studies

The purified protein (see sections 2.14) was mixed with (Montanide ISA-50V, SEPPIC, France) adjuvant in a 1/1.1 (v/v) ratio solution (the amount of the protein used depended on the experimental group). The mixture was vortexed to obtain a stable homogenous emulsion.

In this pilot study, three groups of 6-8 week old female Balb/C mice were used in this animal experiment (see table 2.16). Two groups each consisting of three 6-8 week old female Balb/C mice was injected intraperitoneally (i.p) with the emulsified protein (day one) the third (control) group consisted of two mice and were inoculated with PBS. The two vaccinated groups; a booster dose was inoculated on day 14. A third inoculation was performed on day 28 of the experiment. Blood samples were collected from the tail

vein on day 20 and day 35 of the experiment. The final bleed out from the heart was performed on day 45 of the experiment which terminated the experiment. The blood obtained from the final bleed was centrifuged at 3000rpm for 10 minutes; the serum was separated, placed in tubes and stored at -20°C until used for serum neutralisation tests.

The summary of vaccination protocol is shown in Table 2.16

Group	Species	Substance Delivered	Dose of active substance	Route of Delivery	Frequency of inoculation	Time of Delivery	Volume
1	Balb/C mice	BTV8VP2 Fragment 1,2,3 with Montanide ISA 50	15 µg protein/ mouse of each fragment	i.p.	3	Day 1 Day14 Day28	400 µl
2	Balb/C mice	BTV8VP2 Fragment 1,2,3 with Montanide ISA 50	60 µg protein/ mouse of each fragment	i.p.	3	Day 1 Day14 Day28	400 µl

Table 2-16 Experimental animal groups (pilot experiment)

2.17 : Animal Vaccination and challenge

2.17.1 : Animal Model/s (Vaccination of IFNAR -/- mice and BTV-8 challenge)

Eight groups of 48 IFNAR -/- mice were used. Seven groups were immunised twice, three weeks apart, while the remaining control group was not treated. All of the mice were challenged two weeks after the second vaccination, using a lethal dose (10PFU) of BTV-8 (Belgium/06 isolate). Clinical signs in vaccinated and control mice were monitored for 13 days post challenge (pc) and recorded. Animals that showed severe clinical signs were humanely euthanized using the scoring system described in (section 2.18). All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the Centro de Investigación en Sanidad Animal, INIA, Madrid (CISA). The vaccination and challenge protocol is summarised in Tables 2.17 and 2.18.

Table 2-17 Animal Immunisation and challenge groups *All mice were challenged with 10 pfu of BTV-8 on day 35 of the experiment

Group	Species	Code of Formula	Substance Administered*	Dose of active substance	Route of Delivery	Frequency	Time of Delivery
1	IFNAR(-/-) mice	BTV-8VP2 (F1+F2+F3)+ BTV8VP5+ BTV-6VP7 Mon	Prime and Boost inoculation with BTV-8VP2 Fragment1,2,3 + BTV-8VP5 + BTV-6VP7 with Montanide ISA 50	20 µg of each of F1, F2 and F3 protein + 60µg of VP5 + 60µg of VP7/ mouse	i.p	2	Day1 DAY21
2	IFNAR(-/-) mice	BTV-8VP2 complete BTV8VP5+ BTV-6VP7 Mon	**Prime and Boost inoculation with** BTV-8VP2 complete+ BTV-8VP5+ BTV-6VP7 with Montanide ISA 50	60 µg of VP2 complete +60µg of VP560µg of VP7/ mouse	i.p	2	Day1 DAY21
3	IFNAR(-/-) mice	non treated Control group					
Group	**Species**	**Code of Formula**	**Substance Administered***	**Dose of active substance**	**Route of Delivery**	**Frequency**	**Time of Delivery**
1	IFNAR(-/-) mice	rMVA BTV-8 VP2	**Prime and Boost inoculation with** rMVA BTV-8 VP2	$3x10^7$Pfu /mouse	i.p	2	Day1 Day21
2	IFNAR(-/-) mice	pCI-neo BTV-8 Seg2 rMVA BTV-8 VP2	**Prime inoculation with** Plasmid DNA pCI-neo BTV-8 Seg- 2 **Boost inoculation with** rMVA BTV-8 VP2	70-100 µg/ mouse $3x10^7$Pfu /mouse	i.m i.p	1 1	Day1 Day21
3	IFNAR(-/-) mice	pCI-neo BTV-8-Seg7 rMVA BTV-6 VP7	**Prime inoculation with** DNA pCI-neo BTV-8 Seg- 7 **Boost inoculation with** rMVA BTV-6 VP7	100µg/ mouse $3x10^5$Pfu /mouse of rMVA VP7	i.m i.p	1 1	Day1 Day21
4	IFNAR(-/-) mice	(pCI-neo BTV-8-Seg2+ pCI-neo BTV-8-Seg6 + pCI-neo BTV-8-Seg7) (rMVA BTV-8VP2 + rMVA BTV-8 VP5 + rMVA BTV-6 VP7)	**Prime inoculation with** Plasmid DNA pCI-neo BTV-8 Seg- 2+DNA pCI-neo BTV-8 Seg- 6+ DNA pCI-neo BTV-8 Seg- 7 **Boost inoculation with** rMVA BTV-8 VP2+ rMVA BTV-8 VP5+ rMVA BTV-8 VP7	70-100 µg of each plasmid $2x10^7$Pfu /mouse of rMVA VP2+10^5Pfu /mouse of rMVA VP5+10^5Pfu /mouse of rMVA VP7	i.m i.p	1 1	Day1 Day21
5	IFNAR(-/-) mice	(rMVA BTV-8VP2 + rMVA BTV-8 VP5 rMVA BTV-6 VP7)	**Prime and Boost inoculation with** rMVA BTV-8 VP2+ rMVA BTV-8 VP5+ rMVA BTV-8 VP7	$2x10^7$Pfu /mouse of rMVA VP2+ 10^5Pfu /mouse of rMVA VP5+10^5Pfu /mouse of rMVA VP7	i.p	2	Day1 Day21
6	IFNAR(-/-) mice	non treated Control group					

Species	Fluid	Volume	Collection frequency	Duration of the Experiments	Collection site
IFNAR(-/-) mice	Blood	~200-300 µl	Day 0, 20, 34 then day3,5,7,10,12 and 13 post challenge	2 months	Tail vein and submandibular veins

Table 2-18 Schedule of blood sample collection in challenge experiment

2.17.2 : Collecting blood from mice

Two methods were used for blood collection from mice:

a) **Tail vein:** Bleeding from the tail vein was performed via a tail laceration technique. The mouse was restrained and an incision made in the tail vein with a clean scalpel. A labelled eppendrof tube (containing EDTA for samples used for viraemia or without EDTA for samples used in SNT) was placed under the lacerated area to collect blood. Gentle massage was applied to the tail to increase the volume of the blood obtained from the mouse.

b) **Submandibular veins:** The mouse was held by the scruff of the neck. Using a 20G needle, and with enough force the cheek was poked to create a small stick hole so that drops of blood exuded from the point of penetration. An eppendrof tube (with or without EDTA) was placed to collect blood drops. A sterile gauze compress on the bleeding site for 10-30 seconds was used to stop any bleeding. This method is more humane, allows daily blood collection and a volume of 0.5 ml can easily be obtained (Glade, W. T. et al 2005)

2.18 : Post-challenge, clinical scoring system

The clinical signs in vaccinated and control mice were monitored for 13 days post challenge (pi) scored and recorded using a morbidity scoring system. This system was also used to determine humane end points:

- Coat condition: 0 point (normal glossy and well kept); 1 point (face fur ruffled); 2 points (face and body fur ruffled); 3 points (Lack of grooming Unkempt, thin coat).
- Activity: 0 point (Normal); 1 point (apathy, Isolated but responsive to noise); 2 points (huddled/inactive); 3 points (reluctant to

move resulting in no water or/and food consumption with no response to sound and/or touch).

- Body condition: 0 point (normal); 1 point (thin); 2 points (loss of fat, no growth); 3 (loss of fat and muscle, bones prominent)
- Eye condition: 0 point (normal glistening eyes); 1 point (lacrimation in one or both eyes); 2 points (swelling and/or red eyelids); 3 (conjunctivitis, crusting, unable to open eyes).
- Death: 6 points.
- Mice with a score of 6 or more were humanely euthanized
-

2.19 : Serum neutralisation test (SNT)

2.19.1 : Detecting *BTV-8* neutralising antibodies in immunised mice (Pilot experiment)

Two sterile NUNC 96 well plates were used for 6 samples (in duplicates) tested (one plate is for testing and the second is used as negative control). These plates were marked with a marker pen drawing the outlines of the test onto the plate. Row H is the cell control row and does not contain any virus.

Sera (test or control) was diluted 1:20 with diluent (DMEM+HEPES supplemented with 100 IU/ml of Penicillin, 100ug/ml Streptomycin). All sera were heat inactivated at approximately 56°C for ~ 30 minutes.

Test plate: 100µl diluent was added to all the wells except row A (plate1). 200µl of inactivated test serum was added to wells in row A of the test plates. The samples were serially two fold diluted down the plate, to row H, by transferring 100µl from row A to row B, mixed and 100µl transferred from row B to row C, mixed etc. to the bottom of the plate and the final 100µl discarded.

Virus control plate (a minimum of ½ plate): 100µl diluent was added to wells of rows A to E and 200µl of diluent to row H (this is the negative cell control). 100µl control normal serum was added to wells in row G (negative control) and 100µl of serotype specific control serum (positive control) were added to all wells of row F of the virus control part of the plate. 13ml of the virus is required for 1 plate; a dilution of virus was prepared to give 100 $TCID_{50}$/100µl

Virus control: in order to confirm that 100 $TCID_{50}$ had been added to each well, a 10-fold dilution series from 100 $TCID_{50}$/100µl was prepared to give "10" $TCID_{50}$/100µl,

"1" $TCID_{50}$/100µl, "0.1" $TCID_{50}$/100µl and "0.01" $TCID_{50}$/100µl. (200 µl of the 100 $TCID_{50}$/100µl dilution in 1.8ml of media is used to start of). The dilution of the virus in the control section serves as a control that $TCID_{50}$/100µl was used as virus concentration.

Starting with the control plate 100µl of the "0.01 $TCID_{50}$/100µl" dilution was added to each of the wells in row E of the virus control section, then 100µl of the "0.1" to row D, then 100µl of "1" to row C, then 100µl of "10" to row B then 100µl of "100" to rows A, F and G. Row H is the cell growth control and has no virus added. 100µl of the 100 $TCID_{50}$ dose was added to each well of the test plate.

The plates were sealed with tape and incubated for approximately 1 hour at 37°C followed by an overnight incubation at +4°C. This will allow any antibodies to neutralise homologous virus. The following day: plates were removed from 4°C and placed in CO_2 Incubator at ~37°C (to rebalance pH and heat) for ~ 30 minutes. Cell culture medium was prepared, consisting of RPMI + Hepes supplemented with 100 iu/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine and 5% heat inactivated normal bovine serum, adult or foetal. The cells were removed from a 1x confluent 175 cm2 flask of Vero cells using versene/trypsin and resuspended in an appropriate amount of cell medium. Cells were counted using (Hycor) glasstic slide haemacytometer. A cell suspension was prepared in cell culture medium containing

2 x 105 cells/ml.

The plate seal was carefully removed and 50µl of the cell suspension was added to all wells of the plates taking care not to allow the tips to touch the well contents. Plates were resealed tightly and incubated at approximately 37°C. Plates were examined microscopically for cytopathic effects (CPE) at three or four days and six or seven days post addition of cells. If CPE was observed in the Vero cells of a well then the well was scored as positive. . Titres are assigned as the dilution of serum that will give a 50% neutralisation endpoint, i.e. where one well of the duplicate pair will be positive and one will be negative.

2.19.2 : Detecting BTV-8 neutralising antibodies in immunised mice (Challenge experiment)

Neutralising antibody titres against BTV-8 in serum samples were determined by SNTs. Each sample was screened in duplicate. 50µl of DMEM/Hepes supplemented with antibiotic was placed in seven rows of a 96 well flat bottomed tissue culture

(Nunc) microplate. 2 fold dilutions of sera (from 1:5) were added to 50µl of 100 TCID50 of BTV-8, incubated for 1 h at 37°C followed by overnight incubation at 4°C. The following day Vero cells were added at 2 x 10^5 viable cells/ml in 96 well plates and incubated for 6 days at 37 °C. Plates were examined microscopically for cytopathic effects (CPE) at three or four days and six days post addition of cells. Titres were assigned arithmetically as the inverse of the dilution of serum that gave a 50% neutralisation endpoint and expressed as log10 values.

2.19.3 : Virus titration

96 well tissue culture plates were marked out so that row H is for a cell control and rows A to G (columns 1-6) are for BTV-8 virus. The rows were labelled for each dilution (-4, -4.5, -5, -5.5, -6, -6.5, -7). Using a multichannel pipette 200µl DMEM + 1% pen/strep (sample diluent) was added to row H and 100µl sample diluent added to the remaining wells of the plate. Sterile glass bijoux were prepared with serial log10 dilutions of the virus including half log10 dilutions if necessary, these are labeled as (-1, -2, -3, -4, -5, -6 and -7) for the log10 dilutions and (-4.5, -5.5 and -6.5) for the half log10 dilutions.

For log10 dilutions($-^{1, -2, -3, -4, -5, -6}$ and $^{-7}$) using a pipette 2.7 ml of sample diluent was placed into each bijoux and 0.3ml of the virus was serially transfered respectively of virus between the bijoux.. For half log dilutions ($10^{-4.5}$, $10^{-5.5}$ and $10^{-6.5}$) 2.2ml of sample diluent was pipetted into bijoux and 1 ml of the preceding log10 virus dilution was transfered into it (e.g. 1ml of diluted virus is transferred from bijoux 10^{-4} to bijoux $10^{-4.5}$.....). Using a pipette 100 µl of each virus dilutions was added to the appropriate row starting with the highest dilution to the lowest dilution i.e. from 10^{-7} to 10^{-4}.

Cell media (RPMI + hepes + 1% pen/strep + 1% L-glut + 5% heat inactivated normal bovine serum) was prepared in the appropriate volume (10 ml is sufficient for one plate). The cells from an appropriate number of 175cm^2 flasks of Vero cells were trypsinised using 10 ml of trypsin versin, this was repeated three times, then the flask was placed in the incubator at 37°C for 10 minutes or until the cells are dislodged from the flask. The cells were re-suspended in about 5ml of cell media which was prepared earlier on. The cell suspension was diluted 1/100 in trypan blue (0.5 ml of the cell suspension+ 9.5 ml trypan blue). A cover slip was placed on the haemocytometer and about 15 µl of the diluted cell suspension in trypan blue was placed in each chamber, the numbers of cells were counted in each chamber. A cell suspension containing 2 x 10^5 cells/ml medium is prepared, using (N1V1= N2V2) equation.

50 µl of cells was added to all the wells on the plate. The plates were then sealed with a plate sealer and incubated at 37°C. Plates were read for cytopathic effect for 6-7 days. Spearman-Karber Formula was used to calculate the titre of the virus.

2.20 : Seeding tissue culture plates for immune-fluorescence studies

24 well plates were used. BHK-21 cells were harvested by trypsanisation three times with versine trypsin, followed by 10 minutes incubation at 37 °C. The cells were aspirated and centrifuged at (178 × g for 3 minutes). The pellet was re-suspended in 10ml of growth media [DMEM containing HEPES supplemented with 100U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich)]. 13 mm diameter cover slips (glass for confocal microscopy) were placed into the bottom of a 24 well tissue culture plate and rinsed with 70 % ethanol. Excess ethanol was allowed to evaporate prior to the addition of freshly harvested and diluted cells. A quantity of the diluted cells was added to each well so that a desired confluency (60-70%) was achieved by the following day, this is the level of confluency required for immunolabeling. The following day, the media was removed and a diluent of BTV-8 NET 2007/2008 was prepared in a dilution of 1/10, 300 µl of seed virus was applied to each well. The virus was allowed to absorb for 30 minutes at room temperature prior to the addition of 300 µl of the growth media and incubation at 37°C overnight

2.21 : Cell fixation

The cells on the coverslips were fixed by transferring the coverslip into a fresh well containing 4 % paraformaldehyde (w/v) in PBS. The cells were incubated for 1 hour at room temperature to fix. Fixed coverslips were transferred to PBS in a 24 well plate and stored at 4 °C until processing.

2.22 : Antibody labelling and coverslip mounting

The cells were permeabalised using 0.1 % (v/v) Triton X-100 in PBS for 15 min, washed once in PBS, and blocked for 0.5 h using 0.5% (w/v) PBS/BSA blocking buffer. The coverslips were incubated with an in-house produced polyclonal antiserum (raised in mice injected with 60 µg of the VP2 fragments of BTV-8 NET 2006) at a concentration of 1/100 (v/v) in blocking buffer for one hour. They were then washed three times in PBS, and incubated with anti-mouse secondary antibodies conjugated to Alexa-488 (Molecular Probes, Invitrogen Paisley, United Kingdom) in blocking buffer for one hour. The coverslips were washed three times in PBS, incubated for 10 min in 1 × 4'6'-diamidino-2-phenylindole (DAPI), rinsed in deionised water and mounted onto

glass slides using VECTASHIELD®. Coverslips were imaged using a Leica SP2 confocal microscope.

2.23 : Generation of rMVA/gene of interest

2.23.1 : Generation of MVA virus stocks

T75 tissue culture flasks of DF-1 (a chicken fibroblast cell line) were infected with MVA at moi 0.01 for 1 hour at 37°C in serum free medium. The inoculum was removed and 15ml of 10 % FCS-DMEM was added. Flasks were incubated for 3-4 days until 80-100% cytopathic effect (CPE) is visible. Cells were scraped from the flasks using a cell scraper and the cell suspension pelleted at 1500 x g for 10 minutes. The pellet was resuspended in 1ml serum free DMEM and freeze-thawed three times to lyse the cells. The final stock was then titrated using X-Gal staining plaque assays described in section 2.23.6.1.3 a.

2.23.2 : Preparation of cells

CEF's and DF-1 (a chicken fibroblast cell line) were obtained from Microbiological Services of the Institute for Animal Health (IAH)-Compton at a cell density of 10^7cell/ml in 4°C refrigerated tubes. The content of the tube was placed in a T75 flat tissue culture flask containing 40 ml of 10 % FCS-DMEM (Dulbecco Minimum Essential Medium supplemented with antibiotics and 10% foetal calf serum). The flask was then placed in the incubator at 37°C (±1°C), 5% CO_2 for 24-48 hours to reach 80% confluence.

2.23.3 : Splitting cells

Confluent cell monolayers were grown in tissue culture flasks (2.21.2). After removing the 10% DMEM growth medium they were washed with 10 ml of Hank's balanced salt solution. The Hank's solution was removed from the flasks and Trypsin versene solution was added, using a volume adequate to cover the entire surface of the vessel. The flask was then incubated in the CO_2 incubator at 37°C (±1°C) for 4 minutes. When the cells began to detach from the surface and were then recovered by adding 10 ml of growth medium. The volume of suspension recovered was divided equally between the numbers of new flasks required; suitable volumes of 10 % FCS-DMEM (Dulbecco Minimum Essential Medium supplemented with antibiotics and 10% foetal calf serum) were added to each flask. Flasks were then placed in the CO_2 incubator at 37°C.

2.23.4 : Seeding 6, 12 and 24 well tissue culture plates

6 well tissue culture plates were seeded at the appropriate cell density ($0.4x10^6$ cells/well for TK-BHK or Chicken Embryo Fibroblasts). Alternatively, 12 well or 24 well tissue culture plates might be used using half or ¼ of the cell densities specified above.

The cell suspension prepared as described in (section 2.23.3) was transferred into 50ml sterile tubes and centrifuged at 700g for 5 minutes, 22°C. The cells were resuspended and diluted in growth medium to the desired cell density using a haemocytometer, seeded onto the plates in (DMEM containing 10% FBS and antibiotic if used for plaque assay/ or antibiotic free if used for transfection) and incubated overnight at 37°C (±1°C) in the CO_2 incubator.

2.23.5 a) Transfection of chicken embryo fibroblasts (CEF)

MVA stocks prepared as described in (2.23.1) were used, to infect CEF confluent cells prepared as described in (2.23.2). MVA was freeze thawed three times, used to infect CEF cells at a multiplicity of infection (moi) 0.1 in (10% FBS DMEM that is antibiotic free). The infected plates were incubated at 37°C for 1 hour. Transfections were performed using Lipofectamin 2000 (Invitrogen), according to the manufacturer's instructions. 4µg of the recombinant pSC11 plasmid (pSC11- BTV-8- Seg 2, pSC11-BTV-8 Seg-6 or pSC11- BTV-6-Seg-7) was diluted in 100 µl of antibiotic and serum free DMEM, incubated at room temperature for 5 minutes. 4µl Lipofectamine 2000 (Invitrogen) was diluted In 100 µl of antibiotic and serum free DMEM. The diluted recombinant pSC11 plasmid was mixed with diluted Lipofectamine and incubated at room temperature for 20 minutes. Transfection was performed in 24 well plates using 100 µl of the transfection mixtures. The plates were placed in the incubator at 37 °C in 5 % CO_2 for 4 hours. Transfection mixtures were removed 4 hours later and replaced with new media, placed in the incubator at 37 °C in 5 % CO_2 overnight. The following day, cells were scraped from the wells using a 1ml tip and the cell suspension was centrifuged at 1500 x g for 10 minutes. The pellet is resuspended in 1ml serum free DMEM. This virus is the parental rMVA which was used for plaque assay/purification.

b) Transfection of (Human Embryonic Kidney 293 cells) HEK293 cells

confluent HEK293 cells prepared in similar method to that of CEF were transfected as described in section 2.23.5: a) above with pCI-neo BTV-8 Seg2, pCI-neo BTV-8 Seg6 or pCI-neo BTV-8 Seg7. The following day transfected cells were lysed using lysis

buffer as described in (section 2.24) and used for RNA extraction as described in section 2.3.2

2.23.6 : Selection of recombinant viruses

2.23.6.1 Plaque assay

2.23.6.1.1 : Infection of cells with recombinant MVA

One day before the assay six-well plates were seeded with CEF or TK-BHK cells as described in (Section 2.23.6.1.3 b) one day before the assay was carried out. The cell monolayers in the wells were checked the following day. If cells were evenly distributed and healthy, the plaque assay was carried out. Before doing the assay, sterile 5% LMP low melting point agarose (Invitrogen) (previously prepared by dissolving 5gm of Low melting agarose in 100 ml of water then autoclaved) was melted in a 'microwave' then cooled to 42°C in a water bath.

A transfection / co-infection harvest (parental rMVA or individual plaque picks) from a plaque assay believed to contain recombinant MVA viruses was used to infect the cell monolayers. The MVA sample was freeze-thawed 3 times before doing the plaque assay. Various dilutions of MVA samples were made in serum free DMEM and these dilutions used as inocula.

The growth medium was removed from the wells and 1 ml of serum free DMEM medium was placed in each well. Then 0.2 ml of the samples (virus inoculums) was added to the wells and the plates incubated at 37°C (±1°C) for 1 hour in a CO_2 incubator.

2.23.6.1.2 : Addition of the 1st agarose overlay

Plates prepared as described in (Section 2.23.6.1.1) were removed from the incubator one hour after the addition of the inoculum. 1% LMP agarose was prepared by making a 1/5 dilution of the 5% LMP agarose in 5% DMEM (made by mixing equal volumes of 10% FBS DMEM and serum free DMEM). The 5% DMEM medium was warmed to 37°C before adding the 5% LMP agarose. Once the overlay medium was prepared, the inoculum was removed from the wells and 2-2.5 ml of the overlay was added to each well. The plates were left on a level surface for 10 minutes until the agarose had set. Then they were transferred to the incubator at 37°C (±1°C), 5% CO_2.

2.23.6.1.3 : Addition of the 2nd agarose overlay

a) (Selection of β- Galactosidase positive virus) in CEF infected cells

β- Galactosidase screening is based on the coinsertion of the E. coli *lacZ* gene along with the gene of interest, under the control of a Vaccinia virus promoter, into the vaccinia virus genome; recombinant viruses will make blue plaques in medium containing Xgal (Earl et al., 1998)

Two or three days after the addition of the 1st layer of agarose (as described in section 2.23.6.1.2), a second agarose overlay containing 400 μg / ml of 5-bromo-4-chloro-3-indolyl β-D galactopyranoside XGal (Sigma) was added to the wells. For this, the 5% LMP agarose was melted in the microwave and cooled down to 42°C (as described in section 2.23.6.1.2). 1% agarose overlay containing Xgal was prepared (equal volumes of 10% and serum free DMEM were mixed and warmed up to 37°C, Xgal added to a final dilution of 1 / 100, mixed thoroughly), and then 5% LMP agarose was added to achieve a final concentration of 1%. 1.5 ml of the agarose overlay was added to each well and the plates left on a level surface until the agarose had set. Then the plates were returned to the 37°C (±1°C), 5% CO2 incubator for 24 hours.

b) Selection of recombinant TK- phenotype, in TK-BHK infected cells

Two or three days after the addition of the 1st layer of agarose as described in section (2.23.6.1.2), the second agarose overlay containing 400 μg / ml of 5-bromo-4-chloro-3-indolyl β-D galactopyranoside XGal (Sigma) and BrdU 5-Bromo-2'-deoxyuridine (Invitrogen) was added to the wells. BrdU was phosphorylated and incorporated into viral DNA in the presence of an active TK, which causes lethal mutations whereas if TK- cells are used, then TK- virus will replicate normally in the presence of BrdU, TK+ virus will not (Earl et al., 1998) The 5% LMP agarose was melted in the microwave and cooled down to 42°C. 1% agarose overlay containing the BrdU 5-Bromo-2'-deoxyuridine (Invitrogen) was prepared (equal volumes of 10% and serum free DMEM were mixed and warmed up to 37°C, Xgal was added to a final concentration of 1/100 and BrdU is added to a final dilution of 1 / 500, mixed thoroughly) Then 5% LMP agarose added to achieve a final concentration of 1%. 1.5 ml of the agarose overlay is added to each well and the plates left on a level surface until the agarose had set. Then the plates were returned to the 37°C (±1°C), 5% CO_2 incubator for 24 hours.

c) β-Gal Staining selection of recombinant MVA in CEF

The β -Gal Staining Kit from Invitrogen was used to calculate the plaque forming units, as determine by the percentage of cells transfected with a plasmid expressing lacZ. Twenty-four-well plates were seeded with CEF at a cell density of $2X10^5$ and incubated at 37° C. Twenty-four hr later, or when cell monolayers were confluent, the rMVA viruses used to infect the cell monolayers were freeze-thawed 3 times before use for infecting CEF. Various dilutions of the samples were made in serum free DMEM and these dilutions used as inocula for infection.

Before starting, 10X PBS and 10X Fixative Solutions (provided in the kit), were diluted with distilled water to make 1X solutions. 20 mg X-gal was dissolved in 1 ml DMF to prepare a stock solution and stored at -20°C.

Monolayers of cells infected with rMVA were fixed with 1X Fixative Solution for 20 minutes at room temperature

While the plate is in the Fixative Solution, Staining Solution is prepared a follows:

25 µl Solution A, 25 µl Solution B, 25 µl Solution C, 125 µl 20 mg/ml X-gal in DMF, ml 1X PBS. Plates were rinsed twice with 2.5 ml 1X PBS, 1.5 ml Staining Solution was added to the plate which was Incubated at 37°C for 0.5 to 2.0 hours, or longer until the cells stain blue; rocking the plates occasionally to ensure even coverage of the plate. Cells were checked under a microscope (200 x total magnification) for the development of blue colour. Total cells were counted and blue cells in 5-10 random fields of view. The following formula was used:

$$\frac{\#\ \text{plaques}}{d \times V} = \text{pfu/ml}$$

d = dilution factor: V = volume of diluted virus added to the well.

2.23.6.2 : Plaque picking

Twenty four hours after addition of the second agarose overlay (Section (2.23.6.1.3), blue plaques were visible to the naked eye in the monolayer's. To pick these plaques, sterile 1 ml tip or Pasteur pipettes were used. The pipette was stabbed on the top of the plaque so that the pipette is in contact with the monolayer. By suction, the agarose plug was removed. The contents of the pipette were resuspended in a glass vial containing 0.5 ml of serum free DMEM. A different pipette and different vial were used for each plaque. Plaques are stored at -80°C (±10°C).

2.23.6.3 : Plaque Purification

Picked plaques (Section 2.23.6.2) were used for further rounds of plaque assays (Section 2.21.5). The plaque purification process was repeated until only blue plaques

from recombinant virus were visible after X-gal staining, indicating the stock was free from wild type MVA. This usually required four rounds of plaque assays.

2.23.7 : Recombinant virus titration

Picked plaques (Section 2.23.6.2) were freeze thawed three times to release the virus. Using a seeded 6 well plate (Section 2.23.4) one day before titration, wells were labelled as -1,-2 ..... and –ve control.

0.2 ml of the diluted virus was placed in the corresponding well. The plates were incubated at 37°C (±1°C) for 1 hour in the CO_2 incubator, the inocula was removed and replaced with 1st agarose overlay (Section 2.23.6.1.2) and the second agarose overlay was added 2-3 days later (Section 2.23.6.1.3). The numbers of blue plaques were counted the following day. The viral titre was expressed as plaque forming units (pfu) per ml. To calculate the viral titre, the following formula was used.

$$\frac{\#\ \text{plaques}}{d \times V} = \text{pfu/ml}$$

d = dilution factor V = volume of diluted virus added to the well.

2.23.8 : Measurement of viraemia in mice post challenge with BTV-8 (plaque assay using crystal violet):

The level of viraemia was measured in whole blood (collected in EDTA) on days 3, 5, 7, 10 and 12 pi.12 well plates seeded with Vero cells at $2X10^5$, were incubated with several dilution of blood/PBS starting at 1/10 dilution of each blood sample collected. This was followed by a standard plaque assay (under agarose) as previously described in (section 2.23.6.1.2). On day 5 post inoculation In a MBSC cabinet 2 ml of 10% (w/v) TCA (Sigma) (see AppendixVIII o) was added to each well for 10-15 minutes as a cell fixative. The agar plug is removed with water and 0.5 -1 ml of Crystal violet stain (Sigma) (see appendix VIII p) was then added to each well for 30 minutes to stain the cells. The stain was washed off with a gentle water stream.

2.24 : Preparation of cell lysates for SDS-PAGE.

Six-well plates were plated with 2 x 10^6 CEF cells. The following day (cells formed a confluent monolayer) they were infected with MVA VP2 (MOI of 3). At 24h post-infection the cells were washed with PBS. Cell lysates were prepared using 200µl of ice-cold solubilisation buffer [20mM Tris-HCL, 150mM NaCl, 1% sodium deoxycholate, 1% Tergitol, 0.1%, SDS, 2mM EDTA (supplied by Sigma-Aldrich), supplemented with protease inhibitors (Roche) prior to use]. The cell lysate was

harvested and centrifuged at 17900 x *g* for 15 minutes at 4°C, and the supernatants retained. Supernatant was collected and kept at -20°C for use in western blotting. Lysates were mixed 1:1 with Laemmli sample buffer, heated at 95°C for 5 minutes, and separated by SDS-PAGE on 10% polyacrylamide gels

2.25 : Statistical analysis methods

Differences in the outcome following challenge, between the different vaccine groups, were examined using a Kaplan-Meier survival curves and a log-rank test. Differences in other measures (clinical score, onset of clinical signs, virus neutralising antibody titres, viral RNA levels and viral load) were examined using Kruskal-Wallis tests. If the Kruskal-Wallis test identified significant ($P<0.05$) differences amongst vaccine groups, these were explored in more detail using Wilcoxon tests for pairwise comparison between groups. Non-parametric tests were preferred because of the small group sizes and potential non-normality of the errors.

3 CHAPTER 3
Generating cDNA constructs for BTV vaccines development

Generating cDNA constructs for BTV-vaccines development

3.1 : Introduction

Two different BTV vaccines are currently available commercially: containing either live attenuated virus or inactivated-virus, as vaccine-antigens. However, both vaccination strategies have related safety concerns (see Chapter 1 section 1.6.3 a and b). Since both vaccines raise immune responses to all of the viral proteins, it has been difficult to develop assays to differentiate vaccinated from infected animals (DIVA). Consequently there is urgent need for easily produced, safe, cost effective and stable BTV vaccines that provide sterile protection.

Recombinants 'subunit'-vaccines do not contain the infectious agent itself, and cannot therefore provide a source of the virus that could be transmitted in the field. Since subunit vaccines do not contain or raise immune responses to all of the viral proteins, it is also relatively simple to develop DIVA assays based on one of the missing antigens.

The ultimate aim of this project was to develop a safe and effective vaccination strategy that could be used to protect livestock in Europe and the UK, based on expressed BTV subunit-proteins, DNA vaccine, and/or recombinant MVA. Previous studies have indicated that BTV structural proteins VP2, VP5 and VP7 can generate protective immune responses (Calvo-Pinilla et al., 2009). These proteins were therefore chosen as primary targets for expression studies as subunit-vaccine candidates. The project was also intended to explore differences in protection, generated by injection of bacterial expressed proteins, as compared to in-situ expression using DNA and/or recombinant MVA vaccines.

BTV outer-capsid protein VP2 (encoded by Seg-2) mediates cell attachment by intact BTV virus-particles, and stimulates both neutralising antibodies and cell-mediated immune responses, making it a primary candidate for any subunit vaccination strategy. The smaller outer-capsid protein VP5 (encoded by Seg-6) does not appear to raise a high level of distinct neutralising antibodies when used alone to raise antisera (Roy, 1992a). However, it helps to determine the specificity of the neutralising antibody response (Mertens et al 1987, deMaula et al 2000), and has been reported to enhance the generation of protective antibodies raised to VP2 (Roy et al., 1990). BTV outer core protein VP7 (encoded by Seg-7) can also mediate cell-attachment and penetration of *Culicoides* cells, either in culture or in adult vector

insects (Mertens et al 1996) and has been used successfully in vaccination trials (Wade-Evans et al 1996).

Two vaccine strategies were tested; one was based on the use of BTV proteins expressed in a prokaryotic system, while the second was based on *in situ* protein expression following vaccination with DNA and MVA vaccines. BTV isolates representing two topotypic regions, were used in initially studies for the project. These included 'eastern' strains including: BTV-4 (isolate GRE2000 /01); BTV-9 (GRE1999/06); BTV-16 (GRE1999/13) and BTV-8 (NET2006/04) and 'eastern' strains including: BTV-12 (BRA2002/01); BTV-20 (AUS1975/01) and BTV-21 (AUS1979/02). However, BTV-8 which arrived in the Maastricht region of the Netherlands during the summer of 2006 (represented by isolate NET2006/04) spread to most of Europe causing the largest BTV outbreak on record, and leading to the first recorded BT outbreak in the UK during 2007. BTV-8 infected the naïve and highly susceptible northern European sheep and cattle populations, were infected by this serotype. Therefore, all subsequent work carried out in following stages of the project were based on BTV-8 NET2006/04.

The genomic RNAs of BTV migrate in a characteristic pattern during 1% agarose gel electrophoresis (AGE), with three size ranges designated L (segments 1- 3), M (segments 4-6) and S (segments 7-10) (Attoui et al., 2001; Huismans et al., 1983; French and Roy, 1990; Breard et al., 2003). The pattern of genome segment separation is similar in all BTV serotypes (Maan, S. 2004)

This chapter presents the first step of the project, to generate cDNA copies of BTV-8 Seg-2, Seg-5 and Seg.7 required for preparation of next generation BTV vaccines. Because some of the BTV genome segments migrate closely together, including Seg-2 & 3, Seg-4, 5 & 6, and Seg-7, 8, 9 anchor primer ligation, for cDNA synthesis and RT-PCR amplification, was performed on multiple segments simultaneously (Maan et al., 2007). Electrophoretic analysis was subsequently used to confirm the generation of full- length cDNA copies.

3.2 Materials and Methods

The preparation of structurally intact genomic RNA of BTV is an essential first step. The different virus strains used were obtained from the dsRNA virus collection at IAH Pirbright (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm) then propagated in BHK-21 cells. The virus was harvested, when infected cells showed 100% CPE and the viral dsRNA was extracted using Trizol (Chapter 2, section 2.3.1) The quality of the dsRNA was assessed by 1% agarose gel electrophoresis (AGE) containing 0.5μg /ml ethidium bromide (EtBr) (Chapter 2, section 2.7). Several BTV genome segments co-migrate during AGE, including Seg-2 & 3, Seg-4, 5 & 6, and Seg-7, 8, 9, as shown in (figure 3.1). The required dsRNA bands were re-extracted from the gel and ligated to the anchor primer (Chapter 2, section 2.3.4 a). The ligated segments were re-purified by AGE, removing excess anchor primer, then converted into cDNAs using reverse transcriptase (RT). cDNAs were then amplified by PCR using primer 5-15-1 HyperLadder™ I from (Bioline) and DNA Molecular Weight Marker III (Roche) were used as references for estimation of the size of dsRNAs and DNA after analysis by 1% AGE.

3.3 Results

3.3.1 : Extraction and purification of viral dsRNA from infected cells

BTV-12 BRA2002/01; BTV-20 AUS1975/01; BTV-21 AUS1979/02 and BTV-8 NET2006/04 (from the dsRNA virus collection at IAH Pirbright http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm) were grown in BHK cell monolayers. RNA was extracted from the infected cells showing 100% CPE (usually at 24-48 hours post infection), using Trizol and LiCl precipitation, then analysed by 1% AGE (Figure 3.1). The different genome segments separated according to their molecular weight/size, in order of decreasing molecular weight (top to bottom). All of the BTV isolates used contain ten genome segments which generated the same AGE migration pattern, with co-migration of Seg-2 & 3, Seg- 4, 5 & 6, and Seg-7, 8, 9, in the relatively small gels that were used (Figure 3.1).

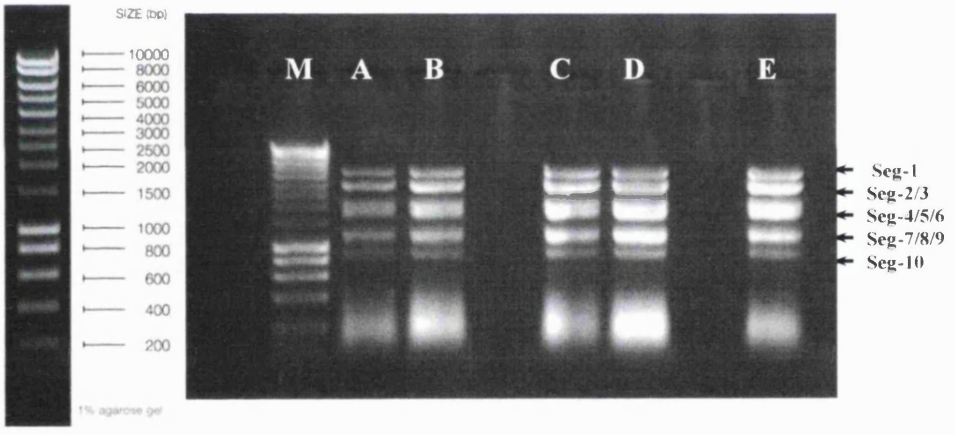


Figure 3-1 AGE analysis of dsRNA extracted from BTV-infected cell cultures

dsRNA was extracted from BTV-infected cell cultures and analysed by 1% AGE. Lane-M shows DNA marker (Hyperladder I); Lane A – RNA of BTV-12 (BRA2002/01); Lane B - BTV-20 (AUS1975/01); Lanes C and D - BTV-21 (AUS1979/02); Lane E - BTV-8 (NET2006/04). The genome segments separate in order of a decreasing molecular weight (top to bottom). Some bands contain more than one segment as indicated.

3.3.2 : Anchor-spacer ligation to BTV genomic RNA

Purified BTV genomic RNAs were ligated to anchor primer molecules at their 3'end, using T4 DNA ligase (New England Bio Labs) (Chapter 2, section 2.5a). The ligated RNA segments were re-purified by AGE as shown for BTV-4 (GRE2000-07); BTV-9 (GRE1999-06); BTV-16 (GRE1999-13) in Figure 3.2, and BTV-12 (BRA2002/01); BTV-20 (AUS1975/01), and BTV-21 (AUS1979/02) in Figure 3.3

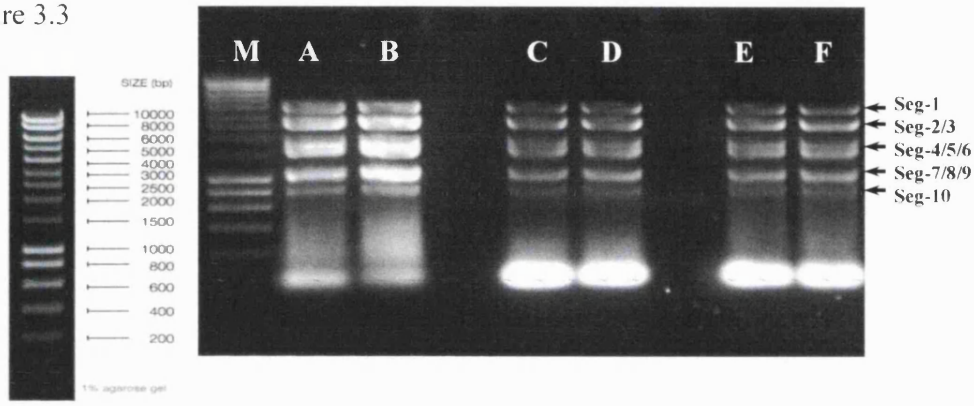


Figure 3-2 AGE analyses of BTV RNA after ligation to the anchor-primer

BTV RNAs were ligated to the anchor primer molecules at their 3'end using T4 DNA ligase then analysed by 1% AGE. Lane-M shows DNA marker (Hyperladder I); Lanes A and B - RNA of BTV-4 (GRE2000 /01); Lanes C and D - RNA of BTV-9 (GRE1999-06); Lanes E and F - RNA of BTV-16 (GRE1999-13) RNA. The migration-pattern / electropherotype of the dsRNAs were the same in all three serotypes.

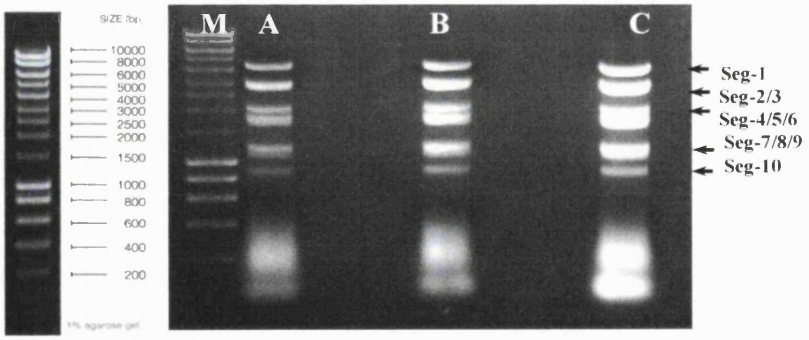


Figure 3-3 AGE analyses of BTV RNA after ligation to the anchor-primer

BTV RNAs were ligated to the anchor primer molecules at their 3'end using T4 DNA ligase then analysed by 1% AGE. Lane-M shows DNA marker (Hyperladder I); Lane A - RNA of BTV-12 (BRA2002/01); Lane B – RNA of BTV-20 (AUS1975/01); Lane C - RNA of BTV-21 (AUS1979/02). The migration-pattern / electropherotype of the dsRNAs were the same in all three serotypes.

3.3.3 : Amplification of full-length segments by FLAC

The BTV genome segments (2/3 and 4/5/6) that had been ligated to anchor primer molecules, then purified on agarose gels, were converted into cDNAs using reverse transcriptase (RT) and primer 5-15-1 (5'-GAGGGATCCAGTTTAGAATCCTCAGAGGTC-3') (Chapter 2, section 2.7). This single primer is complementary to the anchor-primer allowing cDNA synthesis in both directions (from both termini). Amplification of cDNA was carried out by PCR (FLAC method -Maan et al., 2007b). In each case, the amplicons that were generated correspond to full length cDNA segments, as shown in Figure 3.4 for BTV-4, BTV-9 and BTV-16 and in Figure 5 for BTV-12, BTV-20 and BTV-21.

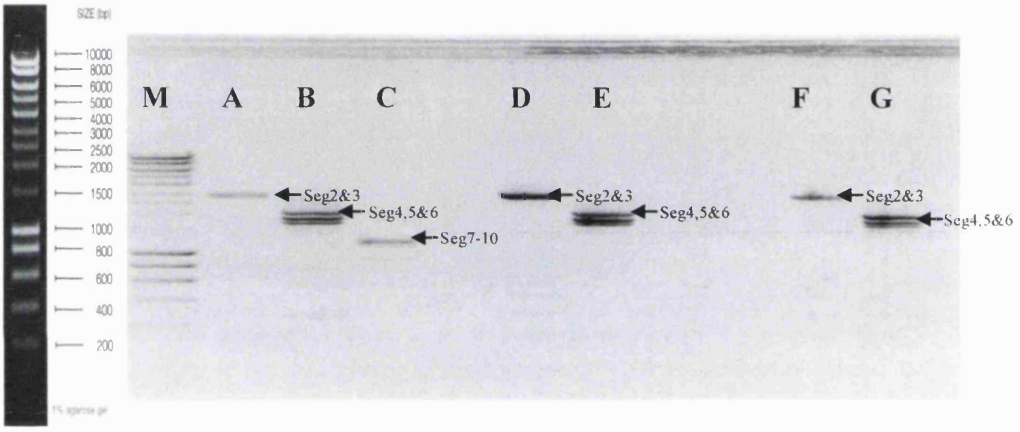


Figure 3-4 cDNA amplicons synthesised by FLAC using different BTV genome segments

BTV genome segments ligated to the anchor-spacer, were converted into cDNAs using reverse transcriptase (RT) and primer 5-15-1, then analysed by 1% AGE. Lane-M shows DNA marker (Hyperladder 1); Lane A - segments 2 & 3 of BTV-4 (GRE2000 /01); Lane B - segments 4, 5 & 6 of BTV-4 (GRE2000 /01); Lane C - segments 7 to 10 of BTV-4 (isolate GRE2000 /01); Lane D - segments 2 & 3 of BTV-9 (GRE1999/06); Lane E - segments 4 to 6 of BTV-9 (GRE1999/06); Lane F - segments 2 & 3 of BTV-16 (GRE1999/13); Lane G - segments 4 to 6 of BTV-16. The amplicons that were generated correspond to full length cDNA segments

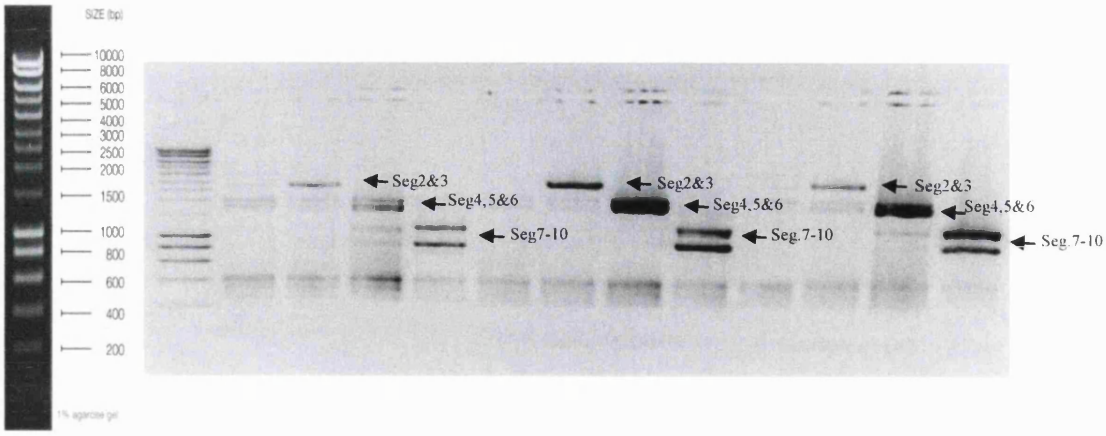


Figure 3-5 cDNA amplicons synthesised by the FLAC method using different BTV genome segments

BTV genome segments ligated to the anchor-spacer were converted into cDNAs using reverse transcriptase (RT) and primer 5-15-1, then analysed by 1% AGE. Lane-M shows DNA marker (Hyperladder I); Lane A shows segments 2 & 3 of BTV-12 (BRA2002/01); Lane B shows segments 4 to 6 of BTV-12 (BRA2002/01); Lane C shows segments 7 to 10 of BTV-12 (BRA2002/01); Lane D shows segments 2+3 of BTV-20 (AUS1975/01), Lane E shows segments 4 to 6 of BTV-20 (AUS1975/01); Lane F shows segments 7 to 10 of BTV-20(AUS1975/01); Lane G shows segments 2+3 of BTV-21 (AUS1979/02); Lane H shows segments 4 to 6 of BTV-21(AUS1979/02); Lane I shows segments 7 to 10 of BTV-21 (AUS1979/02). The amplicons that were generated correspond to full length cDNA segments

3.4 : Conclusion

The migration pattern of BTV genome segments during 1% AGE is dependent on their molecular weights, irrespective of serotype geographical origins (Maan, S. 2004).

The BTV strains used for RNA extraction and cDNA synthesis, represent isolates from two topotypic regions, including 'western' strains (BTV-4, (isolate GRE2000/01); BTV-9 (GRE1999/06); BTV-16 (GRE1999/13) and BTV-8, (NET2006/04) and 'eastern' strains including (BTV-12 (BRA2002/01); BTV-20, (AUS1975/01) and BTV-21 (AUS1979/02).

The entire genome of BTV-8 (NET2006/04) has previously been sequenced (Maan et al., 2008). cDNA copies of the genome segments from other European serotypes, synthesised as described in this Chapter were also sequenced, including Seg-6 of BTV-9 (GRE1999-06), Seg-2 of BTV-16 (GRE1999/13), Seg-6 of BTV-4 (GRE2000/01). The resulting data were aligned to previously published sequences identified using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Seg-6 of BTV-4 (GRE2000/01), BTV-9 (GRE1999-06), BTV-16 Seg-2. Seg-6 of BTV-4 and Seg-2 of BTV-16 (GRE1999/13) were shown to be 99% identical to previously published sequences from the same isolates. Seg-6 of BTV-4 (GRE2000-01) was also 99% identical to that of the reference strain of BTV-4 (RSArrrr/04) derived from a Cypriot strain from the 60s, identified as Asot-1. Considering the geographic proximity of their original isolation sites, this relationship is unsurprising. BTV-9 (GRE1999-06) Seg-6 was 99% identical to that of BTV-9 (SER2001/01), suggesting that both strains are derived from the same outbreak/lineage and therefore share a recent common ancestry.

The sequencing studies conducted in this chapter show that they were authentic copies without insertions/deletions and were therefore suitable for expression studies. However, based on the importance of BTV-8 and the outbreak this serotype caused in Northern Europe with the availability of the complete sequences of Seg-2, Seg-6 and Seg-7 of BTV-8, all work in the following chapters used BTV-8/or BTV-6 constructs

4 CHAPTER 4
Bacterial cloning and expression

Chapter 4: Bacterial cloning and expression

4.1 Introduction

Bacterial systems remain attractive for recombinant expression of viral proteins (Terpe, 2006). The Gram- negative bacterium *Escherichia coli* (*E. coli,)* was first used to express recombinant proteins and remains an attractive system due to its rapid growth, low cost, well-characterised genetics and high-level production of heterologous proteins. (Hannig and Makrides, 1998; Brondyk, W., 2009; Baneyx, F., 1999). This method has enabled the production of therapeutic proteins such as Insulin and Bovine growth hormone (Jana and Deb 2005). Protein expression can even be targeted to specific cellular compartments, although proteins directed to the cytoplasm often leads to the accumulation of insoluble, improperly folded proteins that form as inclusion bodies. However, cytoplasm directed proteins are the most efficiently expressed and this has made them a first choice for heterologous protein production (Hannig and Makrides, 1998; Terpe, 2006; Brondyk, 2009).

The incorporation of recombinant expressed proteins into inclusion bodies, often in an incorrectly folded form, is a significant drawback for bacterial expression, but can sometimes be beneficial, by increasing protein stability and resistance to proteolysis. Refolding schemes are occasionally able to produce active soluble protein from the inclusion bodies (Brondyk, 2009). Several strategies have been used to reduce the chances of inclusion body formation, including lowering the incubation temperature, reducing the rate of protein synthesis, use of a hydrophilic large fusion protein 'tag'(such as glutathione S-transferase [GST]), or co-expression with a folding-'chaperone' protein (Terpe 2006; Hannig and Makrides, 1998). pGEX is a fusion-protein expression-system that features simple affinity-purification of the expressed protein via glutathione S- transferase (GST) fused to the carboxyl terminus of the inserted sequence (Glover and Hames 1995).

This Chapter describes the cloning of Seg-2, Seg-6 of BTV-8 and Seg-7 of BTV-6 into pGEX and the expression of the proteins they encode in *E. coli*. The objective is to express VP2 (either as an intact protein, or as three polypeptide fragments), as well as VP5 and VP7, then test their ability to generate protective immunity in a mouse model.

The large size of the VP2 protein (974 aa long) adds to the difficulty of expressing it in a soluble form. Bioinformatic analyses have indicated that VP2 of BTV was generated by a gene duplication event (concatermerization), and consequently represents an

'intramolecular' dimer. Infectious sub-viral particles (ISVP) of BTV contain a cleaved version of the intact VP2 molecule and are much more soluble than intact virus particles, also suggesting that cleavage of VP2 into fragments may enhance its solubility (Mertens *et al.,* 1996). To reduce the size of the expression target, and potentially increase its solubility, cDNA constructs of Seg-2 were prepared and cloned as three fragments in pGEX4T2 vector allowing VP2 to be expressed, both as the intact protein and as three overlapping fragments. DeMaula et al., (2000) suggested that BTV neutralising epitopes are located in amino acids 199 and 213 (region 1) and between positions 321to346 (region 2) of VP2, in several BTV serotypes. The VP2 fragments were designed so that these regions were not disrupted, in order to keep the neutralising epitopes intact.

The experimental approach was to clone relevant BTV genes into the expression vector (pGEX4T2), under control of the *tac* promoter. To avoid cleavage at the EcoRI site in Seg-2 of BTV-8, whilst still using the EcoRI site for cloning into the plasmid vector and expression of fragment 1 protein, PCR reactions were performed using two different primer pairs (Ping Shih et al., 2008). Both reactions used the same reverse primer, containing a NotI restriction site. However, the forward primers did not contain a restriction site, but target 'footprints' a few nucleotides offset from each other, so that when both PCR amplicons are hybridized, they produce an EcoRI cohesive end at the 5' end of the amplicon. After digestion of the amplicons with NotI the VP2 fragment was inserted into pGEX4T2 using the EcoRI and the NotI sites. The other VP2 fragmented proteins as well as VP5 and VP7 were generated via PCR using gene-specific primers containing EcoRI and NotI restriction sites then cloned into the pGEX vector for expression in E coli.

4.2 : Materials and Methods

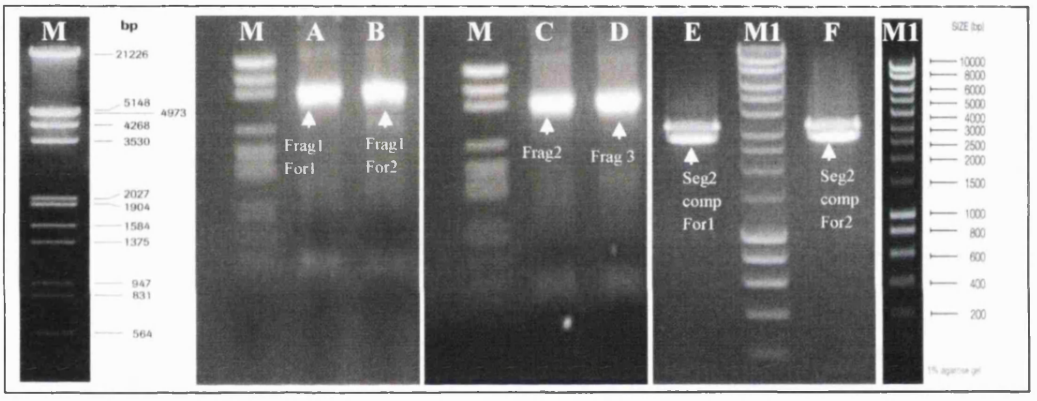
Seg-2 cDNAs were amplified by PCR, then cloned into pGEX4T2 vectors as three fragments, as well as a complete ORF. cDNAs of Seg-6 and Seg-7 were also amplified by PCR then cloned in pGEX4T2 (Chapters 2 Section 2.8.1.2 & Chapter 3). XL1-Blue chemically competent bacterial cells were transfected with the recombinant pGEX4T2 (Chapter 2, Section 2.9). The PCR products obtained from bacterial colonies, with the correct sequence, were used for protein expression in *E. Coli* as described (Chapter 2, Section 2.11.1). Expression using pGEX4T2 generates amino terminus-tagged GST/BTV fusion proteins, allowing them to be purified using immobilised glutathione attached to sepharose beads (Chapter 2 Section 2.11.1). The expressed and purified proteins were analysed by SDS-PAGE and western blots, using anti-GST antibodies raised in rabbits (Chapter 2 Section 2.13).

4.3 : Results

4.3.1 : PCR amplification of BTV-8 Seg-2, Seg-6 and BTV-6 Seg-7 cDNAs

4.3.1.1 : Synthesis of BTV-8 genome segment 2 cDNA fragments and complete construct

The three separate but overlapping fragments, representing the carboxy-terminus, middle and amino-terminus of BTV-8 Seg-2 (fragment 1, fragment 2 and fragment 3 respectively [Figure 4.1a]) and the complete BTV-8 Seg-2 (Figure 4.1b) were amplified from full length cDNAs, using site specific primers (Chapter 2, Table 2.10). This allowed their insertion into pGEX4T2 vectors. All products were amplified successfully (Figure 4.1).



a) b)

Figure 4-1 PCR products of BTV-8 Segment 2

BTV-8 Seg-2 fragment 1, fragment 2 and fragment 3 and complete Seg-2 were amplified from full length cDNAs for cloning into pGEX4T2, using site specific primers shown in table (2.10) then analysed by 1% AGE. Lane-M shows DNA Molecular Weight Marker III (Roche); Lane-M1 shows DNA marker Hyperladder I; Lane (A) shows Fragment-1 forward 1, generated using BTV-8_VP2_Frag1_For1 primer and BTV8_VP2_Frag1_Rev; Lane(B) shows Fragment-1 forward 2 generated using primers BTV8_VP2_Frag1_For2 and BTV8_VP2_Frag1_Rev(A schematic of PCR product preperation of Fragmen1 For1 and For2 are shown in table 2.11); Lane(C) shows Fragment-2 generated using primers BTV8_VP2_Frag2_For and BTV8_VP2_Frag2_Rev; Lane(D) shows Fragment-3 generated using primers BTV8_VP2_Frag3_For and BTV8_VP2_Frag3_Rev; Lane(E) shows BTV8_VP2_complete generated using primers BTV8_VP2_Frag1_For1 and BTV8_VP2_Frag3_Rev; Lane(F) shows BTV8_VP2_complete, generated using primers BTV8_VP2_Frag1_For2 and BTV8_VP2_Frag3_Rev.

4.3.1.2 : Synthesis of BTV-8 genome segment 6 cDNA and BTV-6 Seg-7 construct for cloning purposes

BTV-8 Seg-6 and BTV-6 Seg-7 were amplified from full length cDNAs using site specific primers (shown in Chapter 2: Table 2.12), allowing them to be inserted into pGEX4T2. Seg-6 was amplified as a complete and partial ORF (representing 1200bp - 400bp shorter than the complete segment). All products were amplified successfully (Figure 4.2)

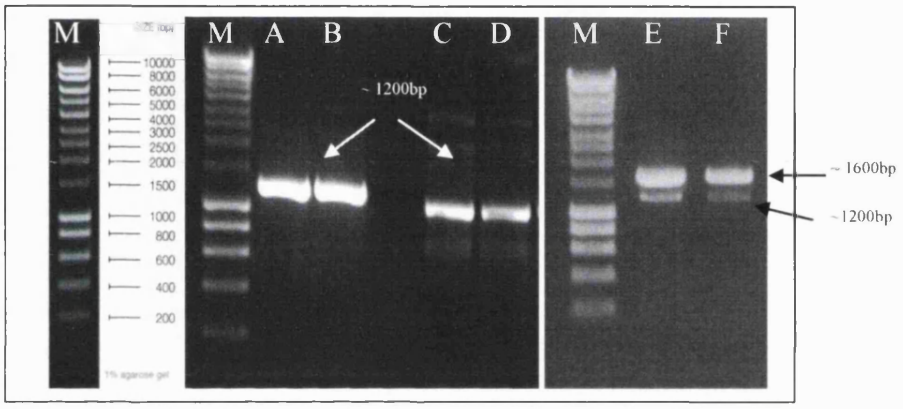
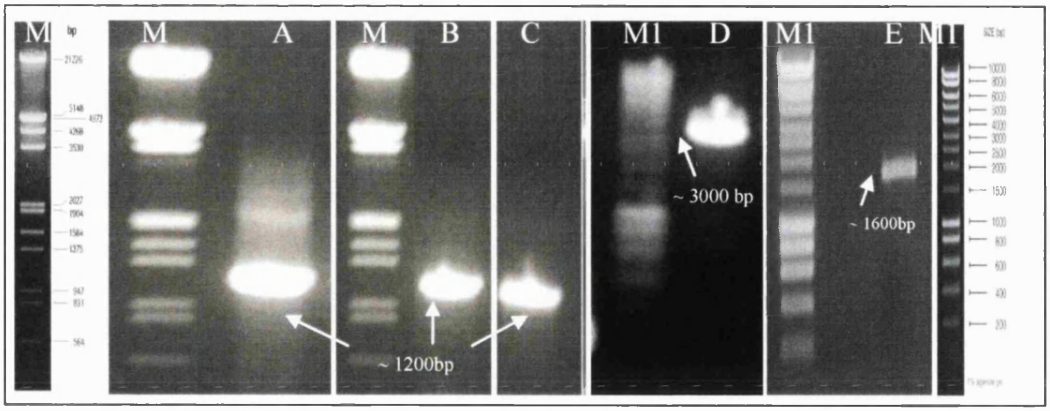


Figure 4-2 PCR products of BTV-8 Segment 6 and BTV-6 Segment 7

Partial Seg-6 and complete Seg-6 of BTV-8, and complete BTV-6 Seg-7 were amplified from full length cDNAs for cloning in pGEX4T using site specific primers (shown in Table 2.12) The cDNA amplicons were analysed by 1% AGE: Lane-M shows DNA marker (Hyperladder I); Lane A and B show BTV-8 partial Seg-6 starting at nt 289 generated using primers BTV8_Seg6_VP5_ For and BTV8_Seg6_VP5_ Rev; Lanes C and D show BTV-6 Seg-7 generated using primers BTV6_Seg7_VP7_ For and BTV6_Seg7_VP7_ Rev; Lanes (E) and (F) shows BTV-8 complete Seg-6 generated using primers BTV8_Seg6_VP5_ For and BTV8_Seg6_VP5_ Rev. Due to the appearance of two bands in the PCR amplification of Seg-6, only the upper band (the expected size of Seg-6)was excised and purified for cloning. All primers are shown in Table (2.12)

4.3.1.3 : Purification of the PCR products prior to restriction digestion

The cDNA products for BTV-8 Seg-2 Fragments 1 (the A of the ATG is position 1, until position 1209), were polished using the *pfx* polymerase to remove the deoxyadenosine residue from the 3' end. The products were mixed, boiled, and purified then analysed by AGE (Figure 4.3a) prior to restriction digestion using NotI (Chapter 2, section 2.8.6.2). The purified products representing fragment-2 and fragment-3 are analysed in Figure 4.3b. The purified complete 'Seg-2' and Seg-6 products are analysed in Figure 4.3c and 4.3d respectively.



a) b) c) d)

Figure 4-3 BTV-8 Seg-2 cDNAs to be inserted into pGEX4T2

AGE analysis of BTV-8 Seg-2 fragment-1 in 'panel a', BTV-8 Seg-2 fragment-2 and 3 in 'panel b', BTV-8 Seg-8 complete in 'panel C': BTV-8 Seg-6 complete in 'panel d'. Lane-M shows DNA marker (DNA Molecular Weight Marker III (Roche)); Lane-M1 shows DNA marker (Hyperladder I); Lane A shows Fragment 1 (produced by mixing the two constructs Fragment1 forward1 -reverse primer and Fragment1 forward2 - same reverse primer – Chapter 2, table 2.11) Lanes B and C show Fragment-2 and Fragment-3 PCR products respectively; Lane D shows mixed btv-8 Seg-2 complete, produced via mixing the two constructs Fragment1 forward1 -reverse primer and Fragment1 forward2 - same reverse primer; Lane F show complete BTV-8 Seg-6. All products showed the expected size.

4.3.2 : Digestion of BTV-8 Seg-2 PCR products (fragments and complete) and pGEX4T2

The PCR amplified cDNA products for Seg-2 (fragments and complete) were digested using EcoRI and/or NotI restriction enzymes, as described in Chapter 2, section 2. 8.6.2. The PCR amplified cDNAs of Seg-6 and Seg-7 were digested using EcoRI and NotI restriction enzymes Chapter 2, section 2.8.6.2. The digested products were analysed by AGE as shown in Figures 4.4 and 4.5.

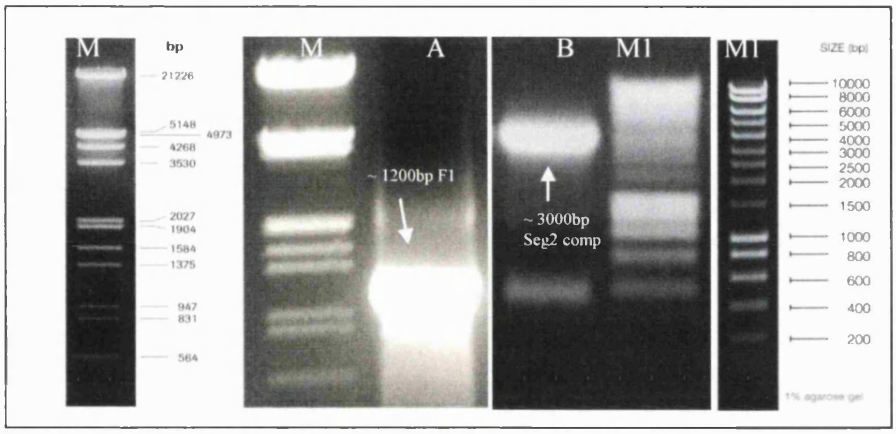


Figure 4-4 AGE analysis of NotI digested cDNA products

BTV-8 Seg-2 fragment-1 and BTV-8 Seg-2 complete PCR products for cloning into pGEX4T2 were digested with NotI. The digested products were analysed by 1% AGE. Lane-M shows DNA marker (DNA Molecular Weight Marker III (Roche)); Lane-M1 shows DNA marker (Hyperladder I); Lane A: shows mixed BTV-8; Seg-2 Fragment-1 PCR products digested using NotI. Lane B shows mixed BTV-8, Seg-2 complete PCR products digested using NotI.

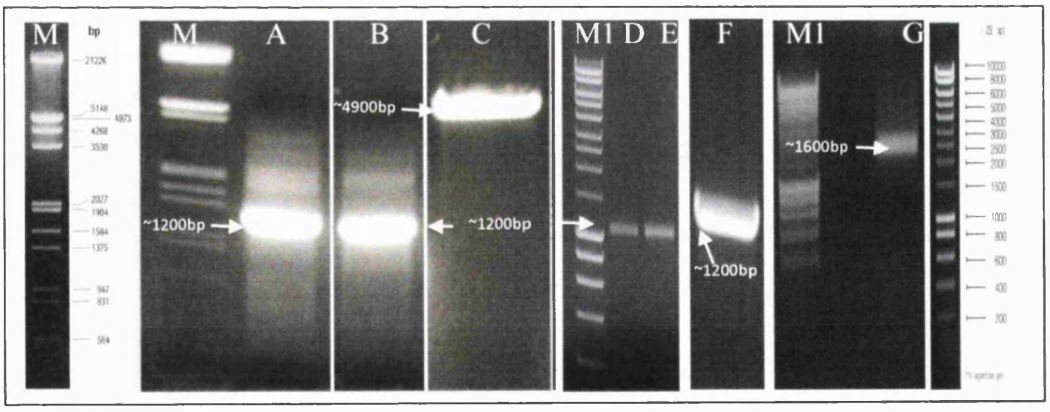


Figure 4-5 Double digestion of PCR products and vector

BTV-8 Seg-2 Fragment 2, Fragment 3, BTV-8 Seg-6, BTV-6 Seg-7 and pGEX4T2 were double digested with NotI and EcoRI for cloning into pGEX4T2. The digested products were analysed by 1% AGE. Lane-M shows DNA marker (DNA Molecular Weight Marker III (Roche)); Lane-M1 shows DNA marker (Hyperladder I): Lane A - digested Fragment 2; Lane B shows digested Fragment 3; Lane C shows the digested pGEX4T2; Lane D and E show digested Seg-7; Lane F shows digested truncated Seg-6 (starting at nt.289) and Lane G shows digested Seg-6 complete. All products showed the expected size.

4.3.3 : Cloning of cDNA fragments and testing of colonies by PCR

The digested fragments were ligated into the appropriately digested vector (pGEX4T2) and transfected into bacteria. XL1- Blue Competent cells (Stratagene) were used for cloning pGEX4T2 recombinant vectors.

Individual colonies (representing the three fragments of BTV-8 Seg-2, complete BTV-8 Seg 2, BTV-8 partial Seg-6, BTV-8 complete Seg-6 and BTV-6 Seg-7 cloned into pGEX4T2 vector) were picked. Only colonies that then showed a good level of turbidity by visual inspection when seeded into LB broth overnight (Chapter 2, section 2.6.4), each colony representing a fragment cloned into its specified vector, were further tested by PCR. The PCR products of fragments 1, 2 and 3 showed expected size of 1200 bp and complete Seg-2 of 3000 bp as shown in Figure 4.6 and Figure 4.7.

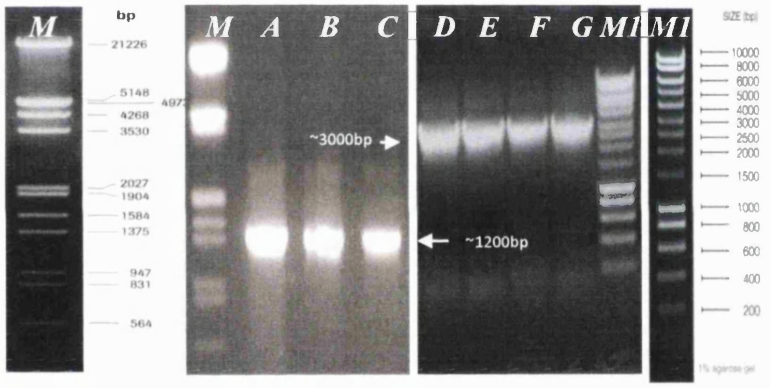


Figure 4-6 Cloning of cDNA fragment and testing colonies by PCR

Colonies obtained from XL1-Blue bacteria transformed with recombinant pGEX4T2 vectors, containing different inserts (Lanes A to G), were tested by PCR and the products analysed by AGE: Lane-M shows DNA marker (DNA Molecular Weight Marker III (Roche)); Lane-M1 shows DNA marker (Hyperladder I); Clones containing Fragment-1 in Lane A; Fragment-2 in Lane B; Fragment-3 in Lane C; complete Seg-2 in Lanes D, E, F & G. Colonies were screened using generic primers pGEX-5' (GGGCTGGCAAGCCACGTTTGGTG) and pGEX-3' (CCGGGAGCTGCATGTGTCAGAGG) see (table 2.8). All products showed the expected size.

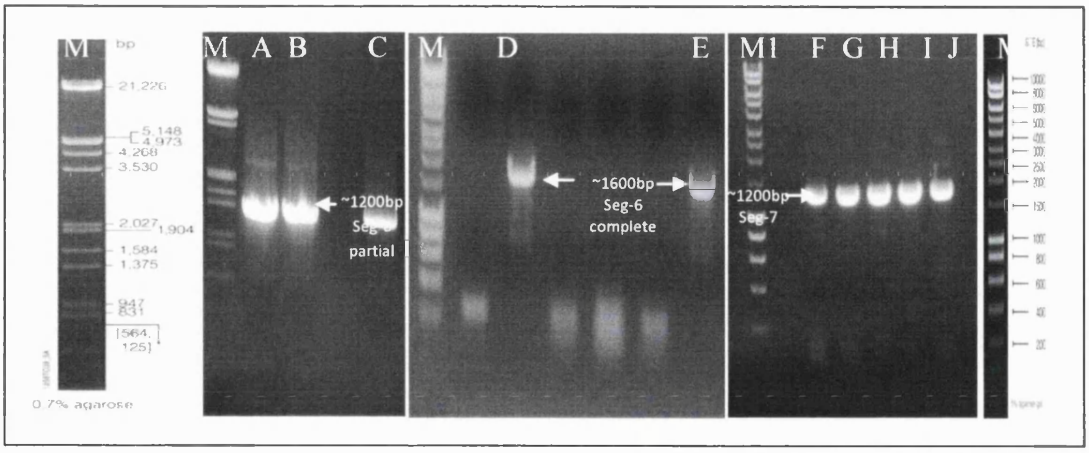


Figure 4-7 Cloning of cDNA and testing colonies by PCR

Colonies obtained from XL1-Blue bacteria transformed with recombinant pGEX4T2 vectors, containing different inserts (Lanes A to G), were tested by PCR and the products analysed by AGE: Lane-M shows DNA marker (Lambda DNA/EcoRI + HindIII Markers (Promega)); Lane-M1 shows DNA marker (Hyperladder I); Clones containing Seg-6 partial in Lanes A, B and C ; Seg-6 complete in Lane D and E; Seg-7 in Lane F, G, H, I and J. Colonies were screened using generic primers pGEX-5' (GGGCTGGCAAGCCACGTTTGGTG) and pGEX-3' (CCGGGAGCTGCATGTGTCAGAGG) see (table 2.8). All products showed the expected size.

4.3.4 : Protein expression of BTV-8 VP2 (fragments and complete), BTV-8 VP5 (partial) and BTV-6 VP7

One of the objectives of this project was to investigate differences in the immune/ protective response following vaccination with fragmented or complete VP2 expressed in a Prokaryotic system. The complete expressed VP2 was insoluble. Therefore BTV-8 Segment-2 was also expressed as three separate overlapping fragments (see Chapter 2 section 2.8.1.1 a) in an attempt to increase solubility of the expressed proteins. Expression of BTV-8 VP5 complete was attempted as described in (chapter 2 section 2.11), however these attempts were unsuccessful (data not shown); thus I expressed the truncated VP5 protein starting at (96 aa position), which corresponds to (nt 289) of the Seg-6 sequence.

The ligated products representing BTV-8 Seg-2 Fragment-1-pGEX4T2, BTV-8 Seg-2 Fragment-2-pGEX4T2, BTV-8 Seg-2 Fragment-3-pGEX4T2, BTV-8 Seg-2 complete-pGEX4T2, BTV-8 Seg-6 partial-pGEX4T2 and BTV-6 Seg-7- pGEX4T2 were transformed into appropriate competent cells (as described in Chapter 2, section 2.9). Positive clones were grown overnight in L.B broth and tested by PCR using plasmid specific primers (specified in Chapter 2, table 2.8), to identify correctly sized products. Sequencing reactions were carried out on the inserts from selected positive colonies

(using at least three correctly sized PCR products for each clone) to verify insertion of the appropriate GST fusion protein in pGEX4T2. Plasmids from bacterial colonies that generated the correct sequence were subsequently used for protein expression (chapter 2 section 2.11). The resulting expressed proteins were analysed by SDS-PAGE (Figures 4.8 and 4.9). All of the proteins were expressed successfully with a size range of 60-75 KDa, corresponding to the expected size of the VP2 fragmented-protein / GST fusion (35-50 KDa of VP2, fused to 26 KDa of GST). However, the expressed proteins (BTV-8 VP2 fragment 1, BTV-8 VP2 fragment 2, BTV-8 VP2 fragment 3) were insoluble (Figure 4.8) and required purification from inclusion bodies (as described in Chapter 2, Section 2.14). Attempts to increase the solubility of the three fragments such as lowering the temperature of expression however, these attempts were unsuccessful (data not shown). BTV-8 VP5 (partial) and BTV-6 VP7 were also expressed in an insoluble form. However, small amounts of soluble protein were also expressed from BTV-8 Segment-6 partial (VP5) and BTV-6 Segment-7 (VP7) (Figure 4.9)

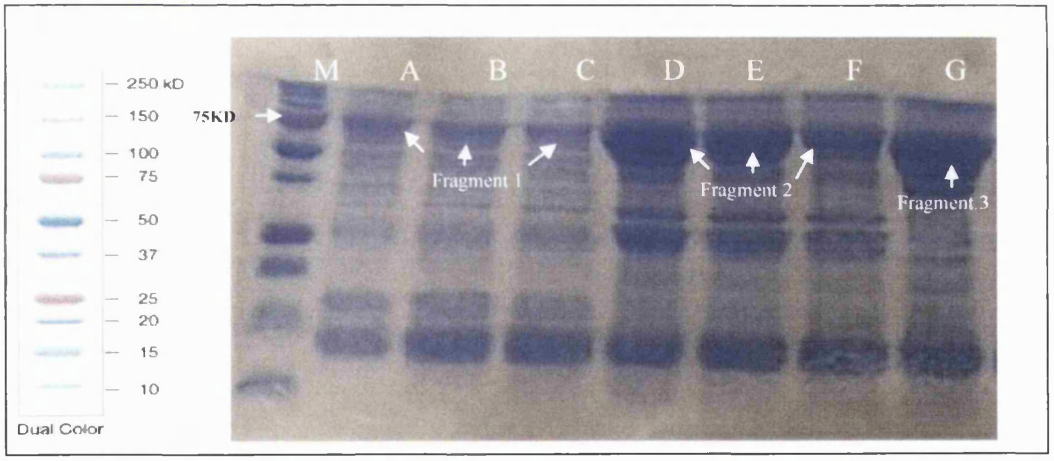


Figure 4-8 SDS-PAGE analysis of bacterial expressed recombinant BTV proteins (Inclusion bodies)

BTV-8 Seg-2 was expressed as three polypeptide fragments using overlapping PCR products from the complete Seg-2, in an attempt to increase solubility. These proteins were analysed by SDS-PAGE and stained in Coomassie Blue. Lane-M shows protein markers (dual colour protein molecular weight marker (BioRad); Lanes A, B and C shows Fragment-1 of BTV-8 VP2; Lanes D, E and F show Fragment-2 of BTV-8 VP2; and Lane G shows Fragment-3 of BTV-8 VP2. The three proteins were expressed in BL-21 cells and had a size of approximately 75 KDa (corresponding to the size of the protein fused to GST protein)

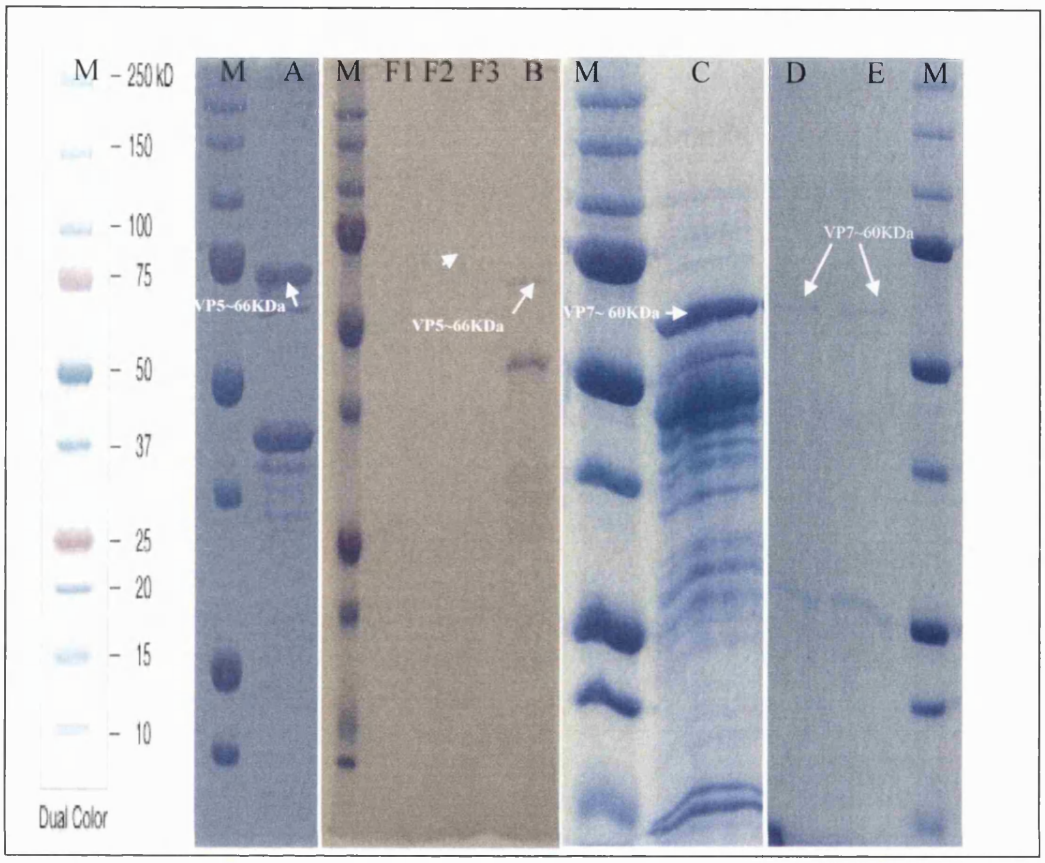


Figure 4-9 SDS-PAGE analysis of bacterial expressed recombinant BTV proteins
BTV-8 Seg-6 (partial) and BTV-6 Seg-7 were expressed in BL-21 bacterial cells, as analysed by SDS-PAGE and stained in Coomassie blue. Lane-M shows protein markers (dual colour protein molecular weight marker (BioRad)) molecular size marker is shown in KDa; Lane A shows BTV-8 VP5 expressed in BL-21 cells (insoluble fraction); Lane F1, F2 and F3 are the lysates of the three fragments of VP2 only traces of soluble F3 were expressed in C41 cell; Lane B shows BTV-8 VP5 protein expressed in C41 cell line (soluble fraction); Lane C shows BTV-6 VP7 (insoluble fraction) and Lane D and E shows BTV-6 VP7 (soluble fraction). The smaller bands found could be degradations products.

4.3.5 : Antigenic reactivity of recombinant expressed BTV proteins

The expressed BTV-GST fusion proteins were analysed by Western-blotting (Chapter 2, section 2.13) using rabbit anti-Glutathione-S-Transferase (GST) antibody (Sigma). Most of the recombinant expressed BTV proteins, with the exception of the complete VP2 protein, were detected by immunoblotting. In each case a protein of the expected size (60-75 KDa) was detected (Figure 4.10). However, only smaller bands (~ 45 KDa) were detected in the soluble fraction of VP5/GST (Figure 4.11). Several bands appeared bellow the expected expressed protein that could be degradation products of the BTV proteins.

The only protein that was not detected by immune-blotting using anti GST was the complete VP2. However, this protein was detected using anti VP2 antibodies raised in mice, as the primary antibody, and a goat anti-Mouse IgG Peroxidase antibody (Sigma). A band of the expected size (as well as smaller proteins that may represent cleavage products, reflecting the known susceptibility of VP2 to proteolytic degradation) was detected (142 KDa corresponding to the size of the VP2 GST fusion protein [116 KDa + 26KDa]) (Figure 4.13).

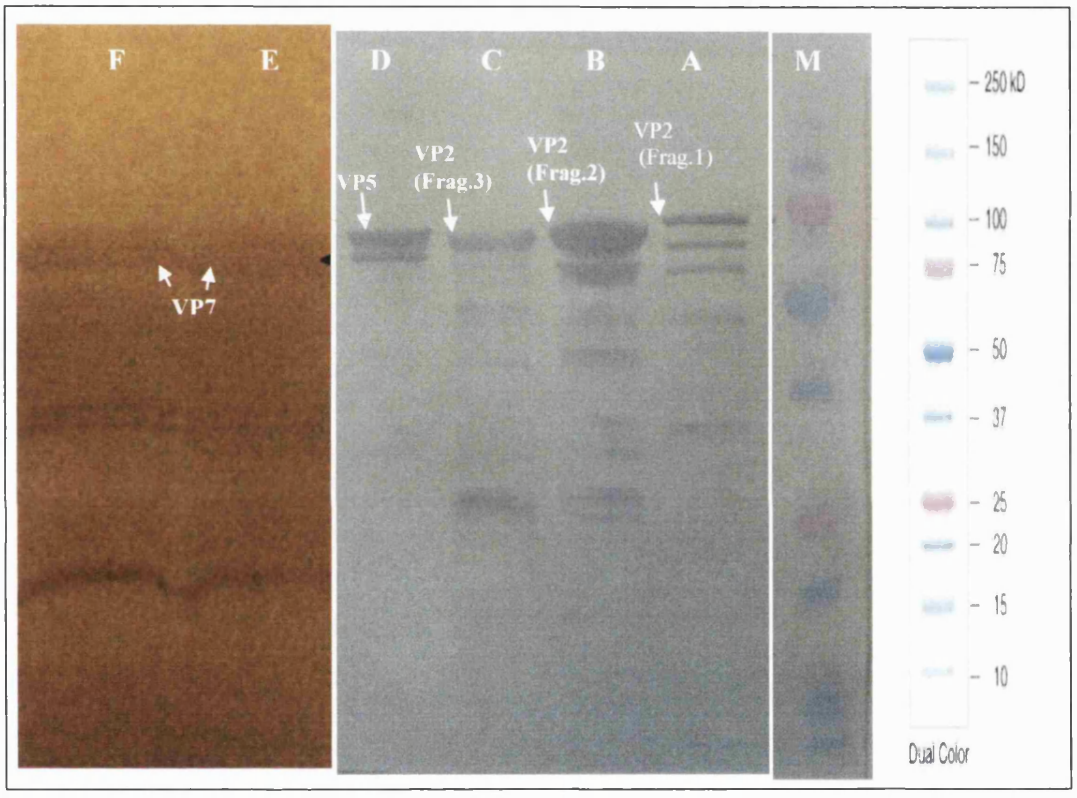


Figure 4-10 Immunoblotting of expressed BTV-8 VP2 fragments, BTV-8 VP5 protein (partial) and BTV-6 VP7

Expressed GST fusion proteins were transferred to nitrocellulose membranes (described in Chapter 2 section 2.13) were detected using anti GST antibody as a primary antibody and anti rabbit antibody as a secondary antibody: Lane (M) shows protein marker (dual colour protein molecular weight marker (BioRad)) molecular size marker is shown in KDa; Lane (A) shows GST/Fragment-1 of BTV-8 VP2; Lane (B) shows GST/Fragment-2 of BTV-8 VP2; Lane(C) shows GST/Fragment-3 of BTV-8 VP2; Lane (D) shows GST/ VP5 of BTV-8; Lanes E and F show GST/VP7 of BTV-6. All proteins were expressed in BL-21 cells and gave their expected sizes (60-75 KDa). All of proteins shown are from insoluble fractions.

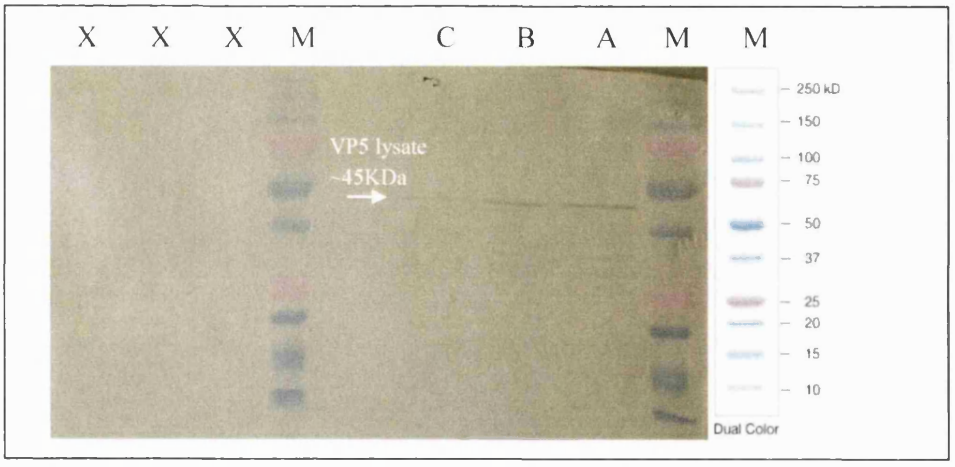


Figure 4-11 Immunoblotting of expressed BTV-8 VP2 fragments, BTV-8 VP5 protein (partial) and BTV-6 VP7

The VP5 (partial)/GST fusion protein expressed in BL-21 cells was analysed by SDS PAGE and transferred to nitrocellulose membrane (as described in Chapter 2, section 2.13). The protein was detected using a primary anti-GST antibody and a secondary anti rabbit antibody: Lane (M) shows protein marker (dual colour protein molecular weight marker (BioRad)) molecular size marker is shown in KDa; Lanes A, B and C show expressed BTV-8 VP5 (partial) protein (soluble fraction); Lane X shows crude VP5 before induction with IPTG (no bands).

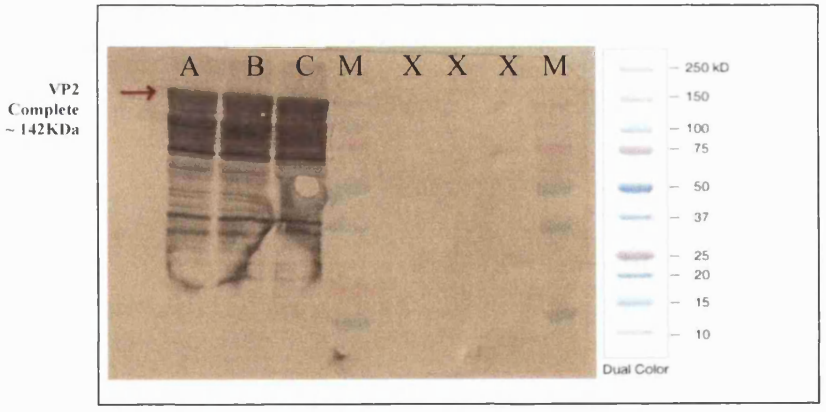


Figure 4-12 Immunoblotting analysis of expressed GST/BTV-8 complete VP2

Expressed GST/complete VP2 fusion protein was transferred to nitrocellulose membrane (as described in Chapter 2 Section 2.13) then detected using a primary anti-VP2 antibody raised in Balb/C mice, and a secondary anti-Mouse IgG Peroxidase antibody (Sigma): Lane (M) shows protein marker (dual colour protein molecular weight marker (BioRad)) molecular size marker is shown in KDa; Lanes A, B and C, contain the GST/BTV-8 VP2 complete protein. The fusion protein was expressed in BL-21 cells and generated bands of the expected size of (~142KDa). Lanes X shows that no soluble (VP2 complete protein) was detected

4.4 : Conclusions

One of the objectives of this project was to test if BTV-8 VP2 expressed in bacteria could be used to induce protective immunity. In order to achieve this objective the VP2 protein of BTV-8 was expressed either as a complete protein or as three separate fragments and then purified for subsequent use in vaccination experiments. This strategy was adapted to improve the chances of obtaining soluble proteins, maintain correct protein folding and preserve the epitope structure of the protein.

cDNA sequences encoding the fragmented and complete VP-2 protein were cloned successfully into pGEX4T2 vector and the proteins were expressed. Several attempts that were made to solubilise the expressed VP2 fragments (including lowering temperature during the expression period, changing duration of expression, use of GST fused promoters and different bacterial cell lines), were unsuccessful in expressing soluble F1 or F2 and only traces of Fragment 3 were expressed using C41 cell line when the expression temperature of 30 °C for 8 hr (Fig. 4.9) The same fragment F3 was also expressed successfully in a soluble form when expressed at 27 °C for 6 hrs. With the complete VP2 presenting a size of approximately 3KDa It was not surprising that VP2 complete would present in an insoluble form.

VP5 and VP7 are much smaller than VP2 and were not intentionally expressed in smaller fragments. Although, a cDNA construct of segment-6 was cloned successfully in pGEX4T2, expression of complete VP5 in bacteria failed. This is probably due to the presence of a hydrophobic regions at the amino terminus of VP5, responsible for interaction with the cell membrane, therefore their removal should reduce the cytotoxic effect of the protein and serve in the expression of truncated protein. This approach was attempted in this project to aid in the expression of BTV-8 membrane protein (VP5) (Brondyk, W., 2009).

cDNA construct of BTV-8 Seg-6 was prepared starting from nt 289 and of BTV-6 segment-7 were cloned successfully in pGEX4T2 and expressed as a soluble GST fusion proteins under the control of tac promoter, a strong promoter induced by Isopropyl-β-d-thiogalactopyranoside (IPTG) (Hannig and Makrides 1998). VP7 and VP5 (truncated) were the only proteins successfully expressed as a soluble protein.

Escherichia coli. was chosen as expression host, due to its ease of use, its well known characteristics and high level of protein expression. One of the major drawbacks in bacterial expression directed to the cytoplasm is the production of insoluble proteins

expressed in the form of inclusion bodies. Several attempts were applied to increase the solubility of the expressed proteins, including growing the bacterial cultures at lower temperatures and fusions with the GST carrier protein to help express mostly soluble proteins ((Glover and Hames 1995; Hannig and Makrides 1998) However, all the expressed VP2 proteins, fragmented or complete, were insoluble. BTV-8 Segment 6 was PCR amplified and showed two bands when analysed by AGE. The smaller band is believed to be as a result of the presence of ATGGGG sequence at (403) region, this is the same sequence as the start of the ORF of Seg-6, possibly leading to some miss priming. In order to obtain the complete Seg-6 only the upper band was excised and purified for Prokaryotic expression. In this project all attempts to express complete VP5 in Prokaryotic system failed. This could be due to the fact that VP5 is a membrane protein and heterologously expressed membrane proteins are often toxic to *E. coli*, preventing cell growth and limiting protein yields (Zoonens and Miroux 2010)

Expression of membrane proteins in bacteria can be difficult due, to membrane-protein targeting (with potentially toxic effects – such as inappropriate membrane fusion) and competition between for high-level expression with other vital host membrane proteins, leading to toxicity and cell death (Zoonens and Miroux 2010). Hassan et al., 2001 reported high helical content in the form of two "amphipathic" helices at amino terminus of VP5 these correlated with cell cytotoxicity and membrane damage, while the same report indicated VP5 was expressed in a soluble form when fused to GST. However, our attempts to express complete VP5 fused to GST were not successful.

(Miroux and Walker 1996; Wagner et.al., 2008) indicated that the use of C41 (DE3) and C43 (DE3) bacterial strains can help to improve membrane protein expression with hardly any toxicity. However, using these strains was also unsuccessful in attempts to express complete VP5 in this study (data not shown). (Brondyk , W. 2009) reported that removing the membrane spanning domain and expression of only the soluble, hydrophilic portion is possible. This approach was applied in this project where the expression of only a part of VP5, starting at aa 97, was amplified by PCR and used for expression. VP5 (partial) and VP7 were expressed successfully as insoluble inclusion bodies using BL-21 (DE3). However, a small amount of both proteins (soluble) were expressed successfully, fused to GST, using the C41 (DE3) cell line. The size of soluble VP5 fused to GST by Western immunoblotting was much smaller than the expected size of approximately (66KDa) equal to (40+26KDa). The size detected using anti GST antibody as a primary antibody, was approximately

(45KDa). Therefore only the insoluble protein was used in animal experiment (see Chapter 5). Leaky expression (a phenomenon associated with use of strong promoters like tac) was suspected in the expression of complete VP5, causing toxicity, however, testing of crude protein before induction showed no bands corresponding to the size of VP5.

5 CHAPTER 5
Animal vaccination with bacterial expressed BTV proteins

Chapter 5: Animal vaccination with bacterial expressed BTV proteins

5.1 Introduction

High levels of protein expression are required for vaccinology studies. This is possible using bacterial expression of recombinant proteins. Together with their simplicity, cost effectiveness, ease of maintenance and growth (which does not require costly media and serum), all, make bacterial systems very attractive (Pathak et al., 2008

Both virus neutralizing antibody and cytotoxic T lymphocytes (CTL) have been shown to have a role in protective immunity against BTV. VP2 is the major immunogen for CTL and the most important BTV protein, in stimulating a neutralising antibody response (Andrew et al., 1995; Roy et al., 2003). Therefore, Seg-2 and the bacterial expression of BTV proteins (VP2) were initially used for vaccinology studies. In early stages of this project a pilot study was conducted to investigate if VP2 expressed as fragments would stimulate neutralising antibodies, A small animal model (Balb-C mice) was used with two different doses (60μg or 15μg/ animal) of three VP2 fragments. The expressed polypeptide fragments were formulated with Montanide ISA-50V adjuvant; an emulsion based on a manide-oleate compound that has been shown to produce antibody levels equivalent to Freund's complete adjuvant (Martinez et al., 1996). The antisera obtained from the final bleed of immunised mice were tested for the presence of antibodies against VP2, via western blotting and Immuno-fluorescence microscopy.

VP5 is believed to enhance the generation of protective neutralising antibodies raised to VP2 (Roy et al., 1990). This suggests that interactions between VP2 and VP5, can affect the conformation of VP2 and consequently its serological properties (Mertens et al., 1989; DeMaula et al., 2000; Roy, 1992a). VP7 can mediate both cell attachment and penetration of BTV in adults Culicoides and in insect vector cells. Antibodies to VP7 can also bind to and neutralise core particles but not fully intact virus (Hutchinson 1999).

In the later stages of this project, an animal experiment was carried out whereby all three major proteins expressed in Bacteria (fragmented VP2+VP5+VP7) formulated with Montanide) were used to inoculate IFN-/- knockout mice, in a prime boost vaccination strategy, at three week intervals, followed by challenge with a lethal dose of BTV-8 two weeks post the boost. A second group of mice was inoculated with (complete VP2+VP5+VP7) in the same manner as used with the fragmented VP2.

These experiments were designed to provide information regarding the protection provided by these proteins when expressed *in vitro,* the difference in immune responses by mice vaccinated with fragmented or complete VP2, and the influence VP5 has on neutralisation of the virus.

The BTV outer core protein VP7 is involved in infection of insect cells and as a serogroup specific antigen could potentially provide cross protection between BTV serotypes (Wade-Evans et al., 1996). Soluble (correctly folded protein) proteins are considered as the best candidates for vaccination trials. However, this project only generated insoluble proteins, which were used in both the pilot experiment and in a final 'challenge' experiment.

This chapter describes both pilot and challenge animal experiments, conducted in a small animal model (Balb/C mice and IFN-/- knockout mice respectively) using recombinant BTV proteins expressed in a Prokaryotic system.

5.2 Materials and Methods

Two animal experiments were designed using recombinant bacterial-expressed BTV proteins for vaccination. In the first experiment (Pilot animal immunisation) Balb/C mice were injected intra peritoneal (i.p) with (fragments 1+ 2 + 3 of BTV-8 VP2) using doses of 60μg or 15μg per animal, formulated with Montanide ISA-50V adjuvant. These formulations were inoculated three times (once every two weeks) and blood samples recovered from the animals on day 45 of the experiment were evaluated using serum neutralisation tests immune-fluorescent microscopy.

A second animal 'challenge' experiment), was conducted using a prime-boost strategy in IFN -/- knock-out mice, inoculated with either fragmented-VP2+ VP5+ VP7, or with complete-VP2+VP5+VP7. Serum recovered at various time points of the experiment were evaluated by virus neutralisation tests. All mice were challenged with a lethal dose of BTV-8, two weeks post boost. Mice were examined daily and clinical signs monitored using a scoring system described in (Chapter 2, section 2.18). Viraemia was also monitored by qRT-PCR and infectious virus was titrated by plaque assay.

5.3 Results

5.3.1 : Inoculation of Balb/C mice with fragmented VP2 (pilot experiment)

15ug or 60ug of BTV-8 VP2 protein (a combination fragment-1, fragment-2 and fragment-3) mixed with Montanide ISA-50V adjuvant (SEPPIC, France), was administered i. p. to Balb/C mice, three times at two week interval. All mice were bled out on day 45 of the experiment. The serum samples obtained from these mice were used for Western blotting and Immune fluorescent microscopy (Figure 5.1 and 5.2)

5.3.1.1 : Immunoblotting using serum obtained from animals inoculated with BTV proteins:

The serum samples obtained from mice inoculated with BTV-8 VP2 protein fragments were tested by Western immuno-blotting, using nitrocellulose strips containing transferred proteins from BTV-8 infected cell-lysates.

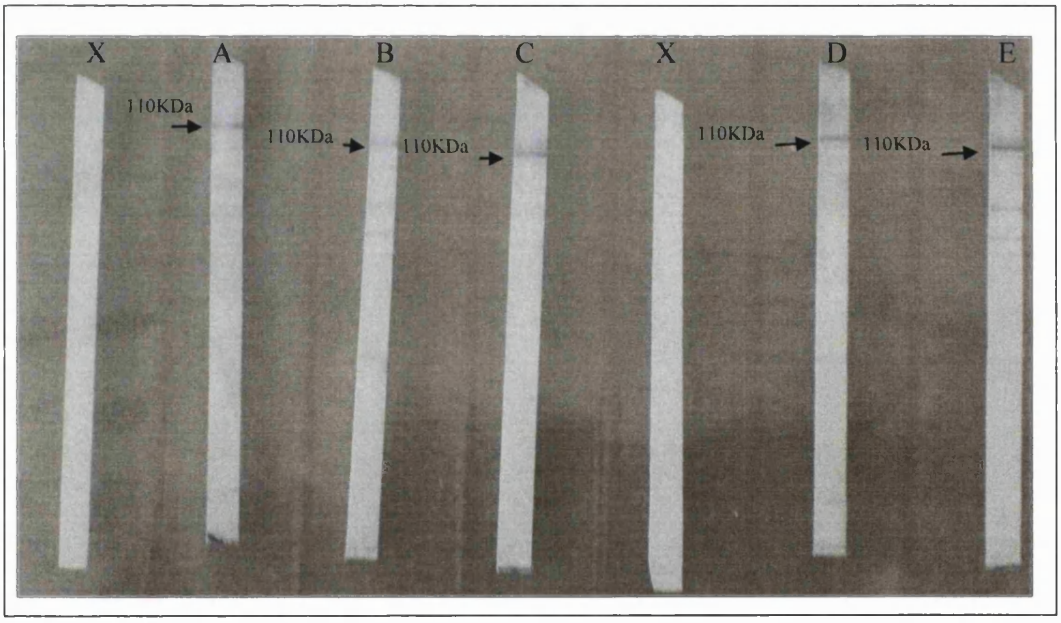


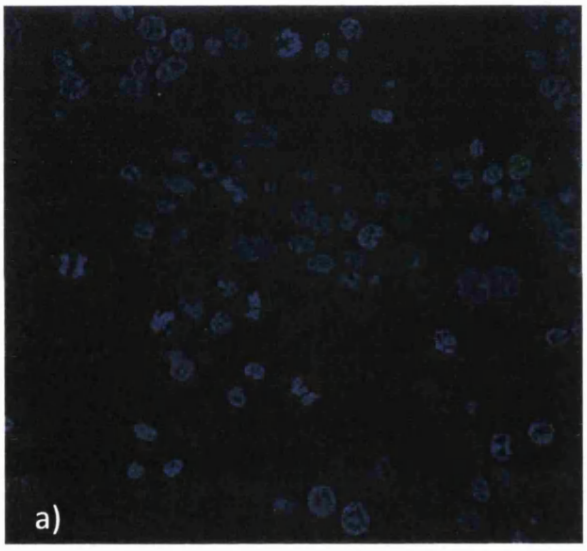
Figure 5-1 Western blot using serum from mice inoculated with VP2 fragments

Lane (X) negative control (mice were inoculated with emulsions containing PBS and Montanide only); Lane A, B and C shows western-blot-strips, using serum from mice inoculated with an emulsion containing 60 µg/mouse of each expressed protein-fragments 1, 2 and 3 of BTV-8 VP2, combined with Montanide (as described in Chapter 2, section 2.15). Lane D and E shows western-blot-strips using serum of mice inoculated with an emulsion containing 15 µg/mouse of each expressed BTV-8 VP2 protein-fragments 1, 2 and 3, combined with Montanide.

The serum was used as a primary antibody and Anti-Mouse IgG (Fc specific)–Peroxidase antibody (Sigma) antibody was used as a secondary antibody (as described in Chapter 2, section 2.13.1). (Figure: 5.1), shows bands at ~ 110KDa equivalent to BTV VP2. These results indicate that BTV-8 VP2 was recognised by serum from mice inoculated with the BTV-8 VP2-fragments.

5.3.1.2 : Immuno-labelling

BHK-21 cells infected with BTV-8 NET/2007 were labelled with the antibodies raised in mice that had been inoculated with expressed BTV-8 VP2 fragments-1, 2 and 3 (60ug) (as described in the previous section).



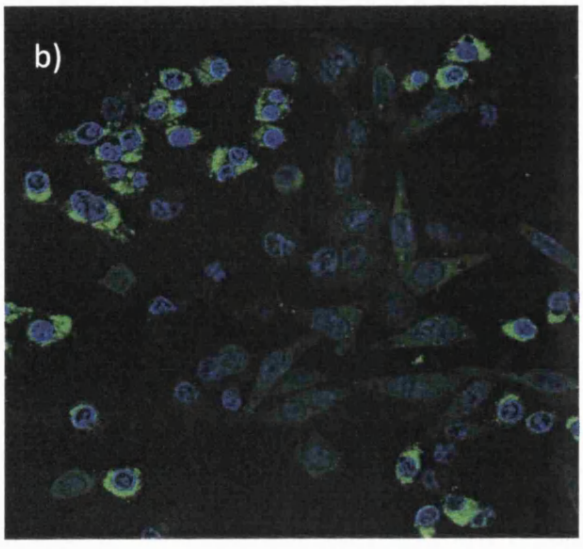


Figure 5-2 Confocal ('confocal laser scanning') photographs of immuno-labelled BHK-21cells

a) Shows a 'negative control', where non-infected BHK-21 cells were treated with antibodies raised in mice inoculated with BTV-8 VP2 protein fragments (1, 2 and 3) as primary antibodies followed by anti mouse antibodies conjugated with Alexa 488 as a secondary antibody. Cell nuclei are stained with DAPI shows in blue.

b) Shows infected BHK-21 cells, which were treated with antibodies raised in mice inoculated with BTV-8 VP2 protein fragments (1, 2 and 3) as primary antibodies. Anti mouse antibody conjugated with Alexa 488 was used as a secondary antibody.
Cell nuclei are stained in blue (DAPI), VP2 is stained in green.

Only BTV 8 infected BHK21 cells shown green fluorescence (Figure 5.2) indicates serum from VP2 immunised mice likely developed antibody against VP2. Therefore, mice inoculated with bacterial expressed VP2 fragments could recognise BTV VP2 as the native protein and induce anti-VP2 antibody.

5.3.2 : The immune / protective response in IFN-/- mice vaccinated with expressed BTV-8 proteins (Challenge experiment)

5.3.2.1 : Post challenge clinical signs

The clinical signs generated post challenge, in vaccinated and unvaccinated-control mice, were monitored up to 13 days post challenge using a morbidity scoring and recording system (Chapter 2 section 2.18). This system provided a basis to determine the protective effect of the different vaccine candidates and was used to determine humane end points.

Mice which developed a score of 6 or more, as well as those that survived to 13 days post-challenge were humanely euthanized (Table 5.1-5.3). All mice in group-3 (unvaccinated control - Table 5.3) developed clinical signs and had to be humanely euthanized on day five post challenge. One mouse in group-1, (vaccinated with fragmented-VP2+VP5+VP7) started to show clinical signs as early as day three post challenge. By day 5 all mice in this group were showing clinical signs and had died, or were humanely euthanized by day 7 post challenge. Although all of the mice in group 2 (vaccinated with complete-VP2+VP5+VP7 - Table 5.2) also showed clinical signs by day five post challenge, only 50% (3/6) had died or were humanely euthanized by day 7 post challenge and the rest survived through to the end of the experiment. The survival rate of each group is shown in figure 5.3. The details of clinical signs of each mouse in each group are summarised in Tables 5.1, 5.2 and 5.3

Animal	day1 p.i	day2 p.i	day3 p.i	day4 p.i	day5 p.i	day 6 p.i	day7 p.i	day 8 p.i	day 9 p.i	day 10 p.i	day 11 p.i	day 12 p.i	day 13 p.i
1.1	No C.S	No C.S	No C.S	1 point (face fur ruffled); 2 points (Huddled/inactive) total=3	1 point (Apathy, Isolated but responsive to Noise); 2 points (face and body fur ruffled) total=3	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
1.2	No C.S	No C.S	No C.S	No C.S	1 point (Apathy, Isolated but responsive to Noise); 2 points (face and body fur ruffled) total=3	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
1.3	No C.S	No C.S	No C.S	No C.S	1 point (face fur ruffled) total=1	*	3 points (Lack of grooming Unkempt, thin coat); 3 points (Loss of fat and muscle, bones prominent); 3 points (Reluctant to move resulting in No water or/and food consumption with No response to sound and/or touch); 3 points (Conjunctivitis, crusting, unable to open eyes). Total=12. This mouse was humanely euthanized	Dead	Dead	Dead	Dead	Dead	Dead
1.4	No C.S	No C.S	No C.S	No C.S	1 point (Apathy, Isolated but responsive to Noise); 2 points (face and body fur ruffled) total=3	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
1.5	No C.S	No C.S	No C.S	No C.S	1 point (Apathy, Isolated but responsive to Noise); 1 point (Lacrimation in one or both eyes); 2 points (face and body fur ruffled) total=4	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
1.6	No C.S	1 point (face fur ruffled) total=1	2 points (face and body fur ruffled; 1 point(Apathy, Isolated but responsive to Noise); 2 points (Swelling and/or Redding eyelids. total=5	1 point (Apathy, Isolated but responsive to Noise); 2 points (face and body fur ruffled) total=3	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead

No C.S= no clinical signs. *= animal not examined

Table 5-1: Clinical signs in Group 1 mice (vaccinated with fragmented-VP2+ VP5+ VP7 expressed in bacteria), post challenge

Mice in group 1 were vaccinated with fragmented-VP2+ VP5+ VP7 expressed in bacteria (Chapter 2, section 2.17.1 and table 2.17), then challenged with a lethal dose of BTV-8. They started showing clinical signs on day 3 post challenge and all mice in this group died or were humanely euthanized by day 7 post challenge.

Animal	day1 p.i	day2 p.i	day3 p.i	day4 p.i	day5 p.i	day6 p.i	day7 p.i	day 8 p.i	day 9 p.i	day 10 p.i	day 11 p.i	day 12 p.i	day 13 p.i
2.1	No C.S	No C.S	No C.S	No C.S	2 points (face and body fur ruffled) total=2	*	1 point (face fur ruffled) total=1	No C.S	No C.S	No C.S	No C.S	No C.S	No C.S
2.2	No C.S	No C.S	No C.S	No C.S	2 points (face and body fur ruffled); 3points (Loss of fat and muscle, bones prominent) total=5	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
2.3	No C.S	No C.S	No C.S	No C.S	2 points (face and body fur ruffled) total=2	*	1 point (face fur ruffled) total=1	No C.S	No C.S	No C.S	No C.S	No C.S	No C.S
2.4	No C.S	No C.S	No C.S	1 point (face fur ruffled). total=1	2 points (face and body fur ruffled); 3points (Loss of fat and muscle, bones prominent) total=5	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
2.5	No C.S	No C.S	No C.S	1 point (face fur ruffled);1 point (Apathy, Isolated but responsive to Noise) total=2	2 points (face and body fur ruffled); 3 points (Loss of fat and muscle, bones prominent) total=5	*	3 points (Reluctant to move resulting in No water or/and food consumption with No response to sound and/or touch) 3 points (Lack of grooming Unkempt, thin coat). 3 points (Loss of fat and muscle, bones prominent) total=9 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead
2.6	No C.S	No C.S	No C.S	No C.S	2 points (face and body fur ruffled) total=2	*	No C.S	No C.S	No C.S	No C.S	No C.S	No C.S	No C.S

No C.S= no clinical signs *= animal not examined

Table 5-2 Clinical signs in Group 2 mice vaccinated with (complete-VP2+ VP5+ VP7 expressed in bacteria) post challenge

Mice in group 2 were vaccinated with complete-VP2+ VP5+ VP7 expressed in bacteria (Chapter 2, section2.17.1 and table 2.17) , then and challenged with a lethal dose of BTV-8 They started showing clinical signs on day 4 post challenge and all mice in this group died or were humanely euthanized by day 7 post challenge.

Animal	day1 p.i	day2 p.i	day3 p.i	day4 p.i	day5 p.i	day 6 p.i	day 7 p.i	day 8 p.i	day 9 p.i	day 10 p.i	day 11 p.i	day 12 p.i	day 13 p.i
3.1	No C.S	No C.S	No C.S	2 points (face and body fur ruffled);2 points (Loss of fat, No growth); 1 point (Apathy, Isolated but responsive to Noise) total=5	3 points (Lack of grooming Unkempt, thin coat); 3points (Loss of fat and muscle, bones prominent). total=6 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
3.2	No C.S	No C.S	No C.S	2 points (face and body fur ruffled) total=2	3 points (Lack of grooming Unkempt, thin coat); 3 points (Loss of fat and muscle, bones prominent). total=6 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
3.3	No C.S	No C.S	1 point (face fur ruffled); 1 point (Apathy, Isolated but responsive to Noise); 1 point (Thin). total=3	2 points (face and body fur ruffled); 3 points (Conjunctivitis, crusting, unable to open eyes). total=5	3 points (Lack of grooming Unkempt, thin coat); 3 points (Loss of fat and muscle, bones prominent); 3points (Conjunctivitis, crusting, unable to open eyes). total=9 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
3.4	No C.S	No C.S	No C.S	2 points (face and body fur ruffled). total=2	3 points (Lack of grooming Unkempt, thin coat); 2 points (Huddled/inactive);1 point (Thin). total=6 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
3.5	No C.S	No C.S	No C.S	2 points (face and body fur ruffled); 1 point (Apathy, Isolated but responsive to Noise) total=3	3 points (Lack of grooming Unkempt, thin coat); 3 points (Loss of fat and muscle, bones prominent)This mouse was humanely sacrificed total=6 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
3.6	No C.S	No C.S	No C.S	2 points (face and body fur ruffled). total=2	3 points (Lack of grooming Unkempt, thin coat); 3points (Loss of fat and muscle, bones prominent).total=6 This mouse was humanely sacrificed	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead

No C.S= no clinical signs *= animal not examined

Table 5-3 Clinical signs in Group 3 mice (unvaccinated control), post challenge

Mice in group 3 (unvaccinated control) were challenged with a lethal dose of BTV-8. They started showing clinical signs on day 3 post challenge and all mice in this group died or were humanely euthanized by day 5 post challenge.

5.3.2.2 : Post challenge survival rates in IFN-/- mice vaccinated with BTV-8 proteins

One of the parameters used to determine the efficiency of bacterial-expressed proteins as vaccines, in this project was based on the survival rate in vaccinated/challenged animals up to 13 days post challenge. Figure 5.3 shows that in group-1 (vaccinated with fragmented-VP2+VP5+VP7), 5/6 mice died on day 6, and the remaining mouse was humanely euthanized on day 7 post challenge. All mice in group-3 (unvaccinated-control) also had to be humanely euthanized, on day five post challenge. However, 50% of the mice in group-2 (vaccinated with complete-VP2+VP5+VP7) survived until the end of the experiment.

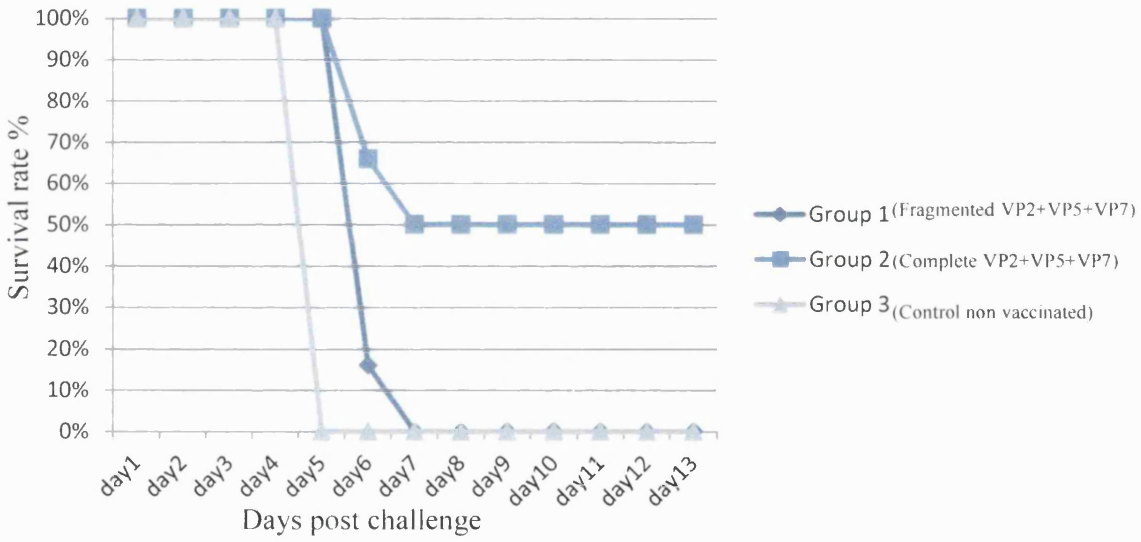


Figure 5-3 Survival rate in IFNAR -/- mice vaccinated with bacterial expressed proteins, then challenged with a lethal dose of BTV-8

Two groups of mice (each consisting of 6 mice), were vaccinated twice, with a 3 weeks interval. Group-1 was vaccinated using a homologous prime boost vaccination, with bacterial-expressed fragmented-VP2+VP5+VP7. Group-2 was vaccinated using a homologous prime boost vaccination with bacterial-expressed complete VP2+VP5+VP7). Group 3 (unvaccinated control) was not treated. All three groups were challenged on day 34 of the experiment (2 weeks post boost-vaccine). Clinical signs and survival rates were monitored for 13 days post challenge.

5.3.2.3 Neutralising antibodies against BTV-8 in IFN-/- mice vaccinated with recombinant - expressed proteins

Virus neutralisation tests were carried out on serum samples taken from vaccinated and control mice, on day 0, day 20, day 34 post vaccination (pv), and on day 7 and 13 pc

(equivalent to days 41 and 47 pv). Figure 5.4 shows that no neutralising antibodies were detected in any group following prime vaccination. No neutralising antibodies were detected in group-1 post boost vaccination, or in the control group (group-3). The only group which showed neutralising antibodies following boost vaccination (starting on day 34) was group-2, which had been vaccinated with bacterial-expressed complete-VP2+VP5+VP7. The levels of neutralising antibodies in this group increased on 7 and day 13 pi. The neutralising antibodies at day 34 represent mean value in group-2. The results on day 7 and day 13 were for the remaining mice which survived (50%) until day7 and 13 respectively.

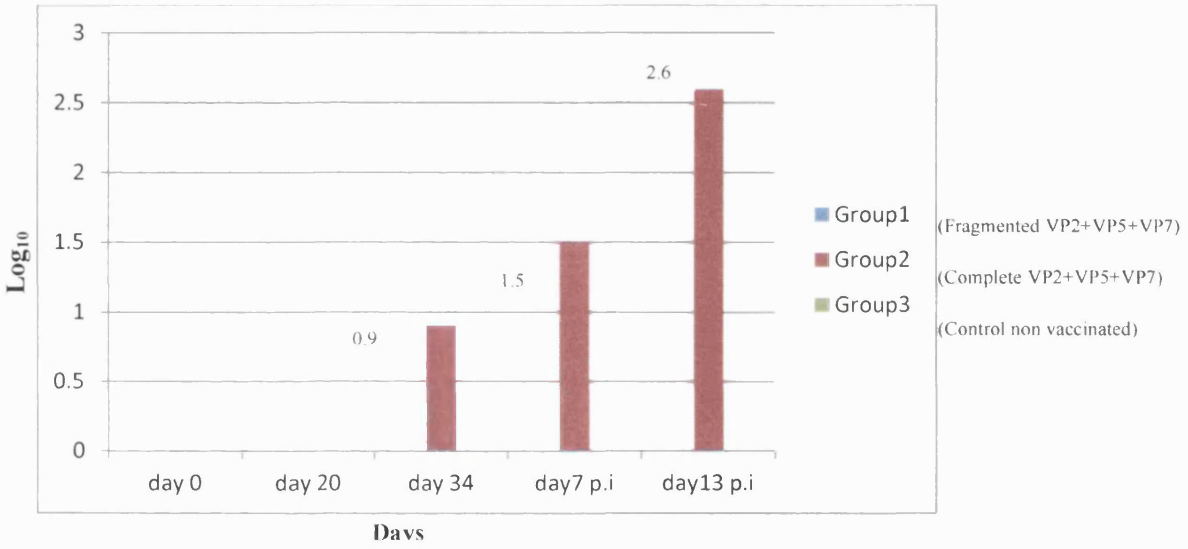


Figure 5-4 Production of neutralising antibodies against BTV-8 in IFNAR -/- mice vaccinated with recombinant (bacterial expressed) BTV-8 proteins

Virus neutralisation tests were carried out on serum samples taken from vaccinated and control mice on day 0, day 20, day 34 post vaccination (pv), and on day 7 and 13 (pi). Group-1 and 2, vaccinated with (fragmented-VP2+VP5+VP7), or (complete-VP2+VP5+VP7) respectively, showed no neutralising antibodies on day 20 (post primary vaccination). No neutralising antibodies were detected in group-1 post booster vaccination, or in the control group (group-3). However, neutralising antibodies were detected in group-2 vaccinated with (bacterial-expressed complete-VP2+VP5+VP7), prior to the challenge on day 34 (2 weeks post boost vaccination). The titre of these antibodies had increased by day 7 and day13 p.i

5.3.2.4 : Immunoblotting using serum obtained from animals vaccinated with BTV proteins (challenge experiment)

Serum samples recovered on (day 34) from mice vaccinated with (BTV-8 VP2-fragments+VP5+BTV6-VP7) (Group1) or (BTV-8 VP2-complete+VP5+BTV6-VP7) (Group2), were tested using nitrocellulose strips containing transferred BTV-8 infected cell-lysate proteins. The serum was used as primary antibody for Western-blotting,

with a secondary anti-mouse IgG (Fc specific)–peroxidase antibody (Sigma) (as described in (Chapter 2: section 2.13.1). Bands of ~110KDa, ~65KDa and ~38KDa (equivalent to BTV-VP2, VP5 and VP7) were detected by the antibodies from group-1, and 2 (unprotected and partially protected mice – respectively). The two smaller bands were not detected by the +ve control serum (from mice inoculated with VP2 alone. These results indicate that all three of the BTV-8 proteins were recognised by antibodies raised in mice inoculated with: expressed VP5 and VP7, and either the combined VP2-fragments or VP2-complete. The lack of protection in group 1 suggests that the antibodies to the complete-expressed-VP2 protein are involved in the protective response shown by group-2, although the response to VP5 and VP7 may also have been required. However, a lack of antibodies to VP7 in the unprotected mice from group 2 (Figure 5.5, Lane B) while those protected in group 2 showed clear antibodies to VP7 (Figure 5.5, Lane B), their presence in the mice that were protected (Figure 5.5 Lane C) suggests that VP7 may also have contributed to the protective response, although variation as a result of technical issues in individual vaccinated animals of Group 2 cannot be completely excluded.

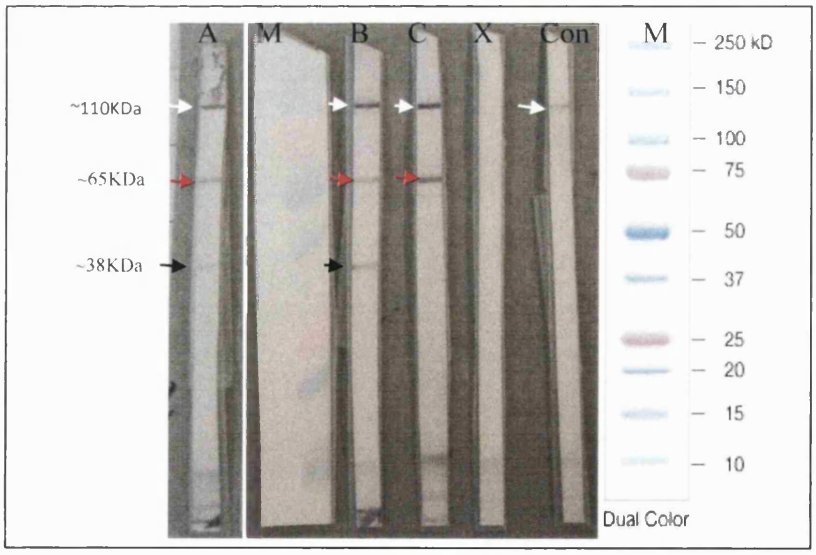


Figure 5-5 Western blotting using serum obtained from mice inoculated with fragmented-VP2+VP5+VP7 or complete-VP2+VP5+VP7, respectively.
Lane-M shows protein markers (dual colour protein molecular weight marker (BioRad)) molecular size marker is shown in KDa; Lane A: was 'developed' serum from mice inoculated with 60 µg of BTV-8 VP2-fragments+VP5+BTV-6 VP7 (group-1). Lanes B and C were developed with serum of mice inoculated with 60 µg of BTV-8 VP2-complete+VP5+ BTV-6 VP7 (group2). In B the serum was recovered from mice which showed protection, while in C serum was recovered from mice which were not protected. Lane (X) negative control (mice not treated - group-3). Lane (Con.)

was developed using serum of mice inoculated with 15 µg VP2 expressed protein fragments-1, 2 and 3as a +ve control.

Groups	Animals	Day3	Day5	Day7	Day10	Day12
	1.1	**31.52**	**25.59**	**dead**	**dead**	**dead**
	1.2	42.19	28.44	dead	dead	dead
Group 1	1.3	No Ct	33.06	25.88	dead	dead
(VP2fragmented+VP5+BT V6-VP7)	1.4	No Ct	31.64	dead	dead	dead
	1.5	33.16	27.3	dead	dead	dead
	1.6	32.14	25.33	dead	dead	dead
	2.1	35.95	No Ct	28.72	30.48	No Ct
	2.2	34.52	28.67	dead	dead	dead
Group 2	2.3	31.7	29.06	31.49	28.41	No Ct
(VP2complete+VP5+BTV 6-VP7)	2.4	40.36	26.65	dead	dead	dead
	2.5	32.09	25.75	26.16	dead	dead
	2.6	No Ct	31.76	30.82	28.68	30.66
	3.1	30.34	25.94	dead	dead	dead
	3.2	32.4	24.82	dead	dead	dead
Group 3	3.3	31.38	24.07	dead	dead	dead
Control	3.4	34.56	28.02	dead	dead	dead
	3.5	No Ct	29.09	dead	dead	dead
	3.6	33.87	26.46	dead	dead	dead

Table 5-4 Detection of BTV-8 RNA in mouse blood at various dates post challenge, by qRT-PCR

BTV RNA was detected in mouse blood post challenge (groups 1, 2 and 3), by RT-qPCR. Viral RNA was detected as early as three days post challenge. Day five was the last day blood samples were taken from the control group (Group3) giving the lowest Ct values (highest levels of RNA) with a mean of 26.4. Results are expressed as Ct values.

5.3.2.5 : Detection of viral RNA in blood post BTV-8 challenge

Blood samples were collected in EDTA from the mice in groups 1, 2 and 3, on days 3, 5, 7, 10 and 12 pi (as described in Chapter 2 section 2.17.2) and BTV RNA was detected by real-time RT-PCR (as described in Chapter 2 section 2.6.3) (Table 5.4). Most mice in the three groups were positive for BTV RNA on day three pi and all of them had a measurable Ct value by day 5pi. The unvaccinated control group (group-3) presented the lowest Ct values (most RNA) with a mean value of 26.4, compared to group-1 (mean value of 28.56) and group-2 (Mean value of 30.31). This indicates some suppression of BTV replication by vaccination with 'fragmented-VP2+VP5+VP7', and a higher level of suppression by vaccination with 'complete-VP2+VP5+VP7, which correlates with the level of protection seen earlier in Figure 5.3.

5.3.2.6 : Measurement of viraemia in mice post challenge with BTV-8

The level of viraemia in the challenged mice (groups 1, 2 and 3) was also measured by plaque assays on days 3, 5, 7, 10 and 12 pi (as described in Chapter 2, section 2.23.8) Table 5.5 shows that no virus was detected in any of the blood samples taken on day 3 pi. However, all of the mice in groups 1 and 3 (vaccinated with fragmented-VP2 +VP5+ VP7, or unvaccinated controls, respectively), had developed viraemia by day 5 pi. The three unprotected mice in group 2 also developed viraemia on days 5 or 7, but only one of the three protected mice in group 2 developed a viraemia at a low level on day 7, that had disappeared by day 10 post challenge.

Groups	Animals	day 3	day 5	day 7	day 10	day 12
	1.1	neg.	$2.5x10^3$	Dead	dead	dead
	1.2	neg.	$6x10^2$	Dead	dead	dead
Group 1	1.3	neg.	$2.5x10^3$	$4x10^3$	dead	dead
(VP2fragmented+VP5+BTV6-VP7)	1.4	neg.	$1x10^2$	Dead	dead	dead
	1.5	neg.	$3x10^3$	Dead	dead	dead
	1.6	neg.	$1x10^3$	Dead	dead	dead
	2.1	neg.	neg.	neg.	neg.	neg.
	2.2	neg.	$5x10^2$	Dead	dead	dead
Group 2	2.3	neg.	neg.	neg.	neg.	neg.
(VP2complete+VP5+BTV6-VP7)	2.4	neg.	$3x10^3$	Dead	dead	dead
	2.5	neg.	neg.	$1x10^2$	dead	dead
	2.6	neg.	neg.	$1x10^2$	neg.	neg.
	3.1	neg.	$6x10^3$	Dead	dead	dead
	3.2	neg.	$5x10^3$	Dead	dead	dead
Group 3	3.3	neg.	$1.2x10^4$	Dead	dead	dead
(Control)	3.4	neg.	$1.5x10^3$	Dead	dead	dead
	3.5	neg.	$6x10^3$	Dead	dead	dead
	3.6	neg.	$2.5x10^3$	Dead	dead	dead

Table 5-5 Detection of BTV-8 infectivity (viraemia) in mouse blood at various dates post challenge

BTV RNA was detected in mouse blood post challenge (groups 1, 2 and 3), by plaque assay. Virus was detected as early as five days post challenge. Day five was the last day blood samples were taken from the control group (Group3) giving the highest average viraemia ($5x10^3$ pfu/ml). Results are expressed as pfu/ml values.

5.4 Conclusion

In the pilot animal immunisation experiment, VP2 (Fragment 1, 2 and 3) were used to raise antibodies in mice, using doses of 60μg or 15μg per animal, formulated with Montanide adjuvant. These formulations were inoculated three times (once every two weeks) and blood samples were taken from the animals. The blood obtained in the final bleed was tested for the presence of antibodies via western blotting and immuno-labelling. Antibodies that were specific for VP2 in western blots, were generated in mice, in response to all three of the expressed VP2 fragments. However, these antibodies failed to neutralise BTV-8 in serum neutralisation tests (SNT) (see Chapter 2 section 2.19). This suggests that the neutralisation epitopes are not displayed on the VP2 fragments, possibly due either to their fragmented nature, or to their expression in a non-native conformation. It is also possible that their association with each other, or with other viral proteins (e.g. VP5 and/or VP7) may influence the neutralising epitopes (DeMaula et al., 2000).

In the challenge experiment, the three fragments of VP2 combined with VP5 and VP7 failed to raise significant levels of neutralisation antibodies. This was reflected by the rapid death of these animals by day 7 pi (Table 5.1). Fragmented VP2 (although the fragments were overlapping thus covering the entire protein), failed to protect IFN-/- knockout mice from a lethal dose of BTV-8 even though combined with VP5 and VP7. The insolubility of the proteins used in the experiment and the choice of the adjuvant (Montanide) may have had an effect on the lack of neutralisation activity of the antibodies produced. Scanlen et al., (2002) concluded that VP2 of AHSV (African horse sickness virus) needs to be soluble to be biologically active and induce a protective immune response in horses. His study also concluded that horses were only fully protected against a lethal challenge when VP2 was administered with saponin adjuvant. However horses were only partially protected when ISA-50 and AlPO4 were used as adjuvants.

One of the objectives of this project was to test the differences in the immune response and protection following vaccination with VP2 (fragmented and complete). Therefore a second group was vaccinated with complete (VP2 expressed in bacteria + VP5 + VP7).

Although, complete VP2 was also expressed as an insoluble protein, it generated neutralising antibodies and provided significantly higher levels of protection 50%. This could be as a result of the partial maintenance of the original VP2 epitopes. There

was some indication that VP7 antibodies were also need for protection in this group, and VP5 may also have played a significant role.

For immunological studies removal of the GST is not required (Harper and Speicher 2008).In this project each protein (Frag.1, frag2, frag.3, VP5 and VP7), were fused to GST protein, this could have impaired the immune response to the antigen of interest which may explain why VP2 complete fused to GST although combined with VP5 and VP7 was more protective, if it was less impaired by the GST.

VP2-complete+VP5+VP7, provided protection in 50% of the vaccinated mice, stimulated neutralising antibodies, which reached their highest levels by day 13 post challenge, possibly due to a further 'boost' caused by the partial replication of the challenge viruses. RT-qPCR specific for BTV Seg-1, performed as described by Shaw et al., (2007) was used to determine post challenge viraemia. This method is highly sensitive and able to detect the presence of just a few copies of the target nucleic acid. However, because the state of circulating BTV nucleic acid is not known (infectious form or inactivated state) (Katz et.al., 1994). A more reliable method was also used to determine the titre of the infectious virus in blood (by plaque assay). Although three of the mice in group 2 died, only one of the three protected mice in (group-2) vaccinated with completeVP2+ VP5+ VP7, had infectious virus as recovered on day 7 post challenge, at a titre of ($1x10^2$ pfu / ml). However, no infectious virus was recovered from these three protected mice on day 10 and 12 post challenge, suggesting that the neutralising antibodies which reached a peak of 2.6 log rate on day 13 post challenge had neutralised the circulating virus that was detected by RT-PCR.

In conclusion VP2 expressed in bacteria as a complete protein combined with VP5 and VP7, although insoluble protected 50% of vaccinated IFN-/- knockout mice when challenged with a lethal dose of BTV-8.

5.5 : Statistical analyses

a) Survival. Survival was significantly higher in group 2 compared with groups 1 and 3 ($P<0.001$).

b) Clinical signs. Significant differences amongst groups in clinical scores were identified on day 4 post challenge (p.c.) ($P=0.03$) and day 5 p.c. ($P=0.003$), but not on day 3 p.c. ($P=0.59$). On day 4 p.c. clinical signs were significantly more severe in group 3 compared with group 2 ($P=0.008$). On day 5 p.c. clinical signs were significantly more severe in group 3 compared with both group 1 ($P=0.003$) and group 2 ($P=0.003$).

c) Virus neutralising antibody titres. A significant difference in antibody titres was identified amongst groups on day 34 post vaccination (p.v.) (P=0.03), day 7 p.c. (P=0.04) and day 13 p.c. (P=0.04), with higher titres in group 2 compared with groups 1 and 3.

d) Viral RNA levels. No significant differences in Ct values amongst groups were found on day 3 p.c. (P=0.60), day 5 p.c. (P=0.22) or day 7 p.c. (P=0.16).

e) Viral load. A significant difference in viral load (titre) amongst groups was identified on day 5 p.c. (P=0.004), with titres in group 3 higher than titres in groups 1 and 2 and titres in group 1 higher than titres in group 2 (all $P<0.05$). However, no significant difference amongst groups was identified on day 7 p.c. (P=0.14).

6 CHAPTER 6
Generation of DNA and recombinant MVA vaccines

Chapter 6: Generation of DNA and recombinant MVA vaccines

6.1 Introduction

DNA immunisation represents a novel approach to vaccination and immunotherapeutic development. Injection of plasmid DNA encoding a foreign gene of interest under the copntrol of a relevant promoter, can result in the subsequent expression of the foreign gene products and the induction of an immune response within the vaccinated host. This is relevant to prophylactic and therapeutic vaccination strategies when the foreign gene represents a protective epitope from a pathogen (Watts and Kennedy, 1999).

DNA vaccines can be based on bacterial plasmids that have been engineered to express the disease-specific antigen, using promoter elements that are active in mammalian cells. They can also be engineered to contain a transcription 'terminator' that is effective in mammalian cells and a selectable marker to facilitate production of the plasmids in transformed bacterial cells. These vaccines can be delivered to the recipient by a variety of routes, including 'gene gun', or intramuscular (IM) injection. The DNA is then taken up by host cells and transcribed into mRNA, from which the vaccine proteins are translated. The antigen is produced within the cells of the vaccinated individual using the host's transcriptional and translational machinery (Dunham, 2002; Anderson et al., 2007). Although there is variability in the success of DNA vaccines to stimulate protective immunity (More and Hill, 2004), they seem to be efficient in heterologous prime-boost vaccination regimes (DNA/rMVA), providing complete protection against a wide range of pathogens, including bluetongue and African horse sickness (Calvo-Pinilla, E., et al., 2009; Castillo-Olivares et al., 2011).

In recent years recombinant live viruses expressing foreign antigens have also been used as antigen delivery systems. Poxvirus vectors offer certain advantages as vaccines, making them one of the most attractive of these viral vectors (Esteban 2009). Indeed, recombinant Vaccinia virus, recombinant Canarypox virus, recombinant capripox virus, have previously been used successfully as gene delivery systems for BTV or AHSV-vaccination (Boone et al.,, 2007; Wade-Evans et al., 1996; Savini et al., 2008; Calvo-Pinilla, E., 2009; Castillo-Olivares, J., 2011).

Chorioallantois vaccine virus Ankara (CVA) was passaged over 570 times in primary chick embryo fibroblast cells (CEF) leading to attenuation of its replication and virulence characteristics and the formation of a new virus that has been named 'Modified Vaccinia Ankara' strain (MVA) (Mayr et.al., 1978; Esteban, M. 2009). As a result of these modifications, MVA is now recognised as a promising and safe vector

for vaccine-antigen delivery, with a well established history and safety record as a vaccine-vector for infectious diseases and malignancies that activates both branches of the immune system (Ramirez et.al. 2000; Esteban, 2009; Kennedy and Greenberg, 2009). This chapter describes the construction of DNA and rMVA vaccine candidates, for use in vaccination and challenge studies of IFN-/- knockout mice.

6.2 : Construction of DNA vaccine plasmids

The basic design of a DNA vaccine requires cloning of the target gene into a mammalian-expression vector. We wanted to develop a DNA vaccine that prevents infection via stimulating neutralising antibodies, therefore the first choice was Seg-2, Seg-6 and Seg-7 of BTV-8 (these three segments encode the three major BTV proteins that stimulate a neutralising-antibody response – see Chapter 1 section 1.10.1). cDNA copies of the relevant ORFs were inserted into the pCI-neo Mammalian Expression Vector, under the human cytomegalovirus (CMV) immediate-early enhancer/promoter region (pCI-neo, Promega). The CMV enhancer/promoter promotes constitutive expression of cloned DNA inserts in mammalian cells.

6.2.1 : Materials and Methods

pCI-neo Mammalian Expression Vector (Promega), containing the cytomegalovirus promoter was used for construction of the DNA vaccine candidates. The plasmids pBRT7 BTV-8 Seg-2; pBRT7 BTV-8 Seg-6; pBRT7 BTV-8 Seg-7, which encode VP2, VP5 and VP7 respectively, were kindly provided by Dr. Andrew Shaw (AMRG-IAH) and were used as a source of genes used to clone into pCI-Neo, to generate: pCI-neo BTV-8 Seg-2; pCI-neo BTV-8 Seg-6; pCI-neo BTV-8 Seg-7. The open reading frame of each segment was amplified by PCR, using primers (described in section 2.8.5.3, table 2.14) designed to insert the XBAI restriction site in the 5′ end and the NOTI restriction site in the 3′ end of the PCR product. The PCR amplicons were subsequently digested and ligated into (XBAI and NOTI) digested pCI-neo using conventional methods as described in section 2.8.6.2. The plasmids generated were analysed by agarose gel electrophoresis and clones were screened by sequencing using pCI-neo specific primers (described in section 2.6.4 and shown in table 2.8), to identify the correct insert.

The recombinant pCI-neo plasmids were subsequently tested for protein expression using specific antibodies. In addition, transcription of the BTV-genes encoded in the plasmid was assessed by RT-PCR with BTV-gene specific primers using RNA extracted from cells transfected with recombinant pCI-neo.

6.2.2 Results

6.2.2.1 : PCR of BTV-8 Seg- 2, Seg-6 and Seg-7 for cloning into pCI-neo

BTV-8 Seg-2, Seg-6 and Seg-7 ORFs were amplified from full length cDNAs using site specific primers (as described in Chapter 2, section 2.8.5.3, Table 2.14). All products were amplified successfully (Figure 6.1), allowing them to be inserted into the pCI-neo vector.

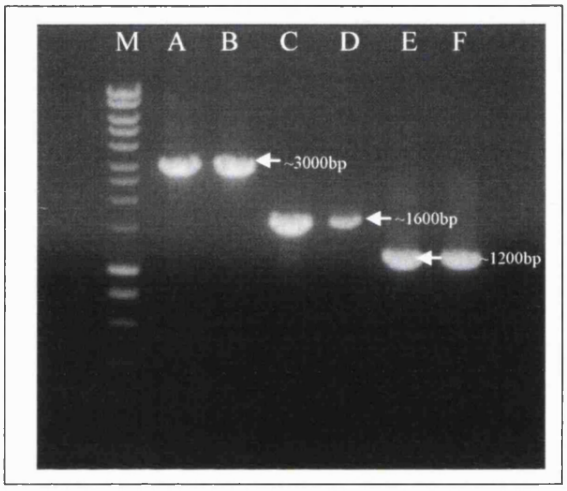


Figure 6-1 PCR of BTV-8 Seg-2, Seg-6 and Seg-7 for insertion into pCI-neo

BTV-8 Seg-2, Seg-6 and Seg-7 were amplified from full length cDNAs, for cloning into pCI-neo, using site specific primers, then analysed by 1% AGE: Lane-M shows DNA marker (Hyperladder I); **Lanes** A and B: show amplified cDNAs for BTV-8 Seg-2, these are replicates of the same sample; Lanes C and D show amplified cDNAs for BTV-8 Seg-6, these are replicates of the same sample. Lanes E and F show amplified cDNAs for BTV-8 Seg-7, these are replicates of the same sample. All primers used are shown in Table 2.14

6.2.2.2 : Digestion of pCI-neo and PCR products for BTV-8 Seg-2, Seg-6 and Seg-7

The PCR amplified cDNAs for Seg-2, Seg-6 and Seg-7 were digested using XBAI and NotI restriction enzymes (as described in Chapter 2, section 2.8.6.2). The digested products were analysed by AGE as shown in Figure 6.2.

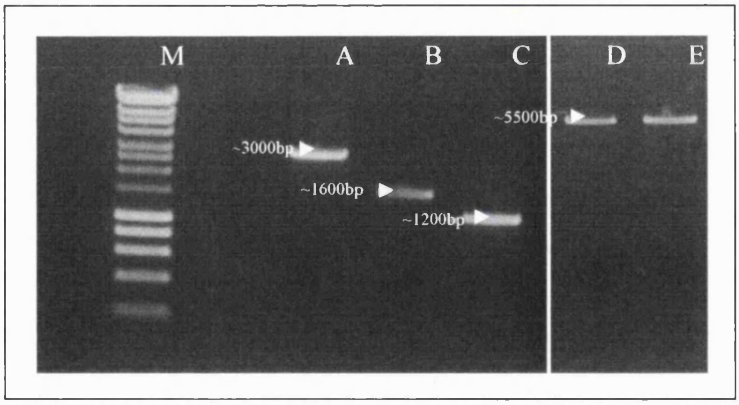


Figure 6-2 AGE analysis of XBAI and NotI digested cDNA products

BTV-8 Seg-2, Seg-6 and Seg-7 PCR products were digested with XBAI and NotI for cloning into pCI-neo. The digested cDNAs were analysed by 1% AGE. Lane-M shows DNA marker (DNA Molecular Weight Marker (Hyperladder I) Lane A shows digested cDNA of BTV-8 Seg-2; Lane B shows digested cDNA of BTV-8 Seg-6; Lane C shows digested cDNA of BTV-8 Seg-7; Lane D and E show XBAI and NotI digested pCI-neo.

6.2.2.3 : Colony screening *E. coli* cultures for plasmid inserts, using PCR

A few colonies from each transformation were picked using a pipette tip and seeded into 200µl LB broth (as described in Chapter 2, section 2.6.4). Samples of the overnight liquid cultures, were added into a PCR mixture containing pCI-neo /F and pCI-neo /R primers. The PCR reactions were carried out then analysed by AGE, as described in Chapter 2 (section 2.7) Figure 6.3 identifies colonies that contain recombinant vectors with inserts of the right sizes. The PCR primers used are shown in (Table 2.8).

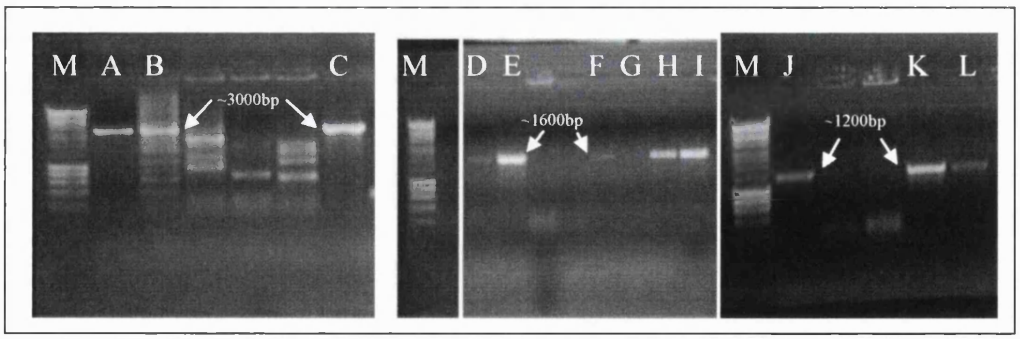


Figure 6-3 Cloning of cDNA in pCI-neo vector and testing colonies by PCR

Colonies obtained from transformations in XL1-Blue bacteria were tested by PCR using pCI-neo /F and pCI-neo /R primers and the products analysed by 1% AGE: Lane-M shows DNA marker (DNA Molecular Weight Marker Hyperladder I); Lanes A, B and C show BTV-8 Segment 2 amplicons; Lanes D, E, F, G, H, I show BTV-8 Seg-6 amplicons; Lanes J, K and L show BTV-8 Seg-7 amplicons. Primers pCI-neo/F and pCI-neo/R primers were used for colony screening PCR. Details of primers used are given in (Table 2.8).

6.2.2.4 : Plasmid Minipreps (DNA vaccines)

Sequencing (colony screening) was used to verify insertion of the correct sequence, into pCI-neo, for BTV-8 Seg-2, BTV-8 Seg-6 and BTV-8 Seg-7. Clones with the complete sequence were selected and used for minprep preparation (DNA vaccines) shown in Figure 6.4.

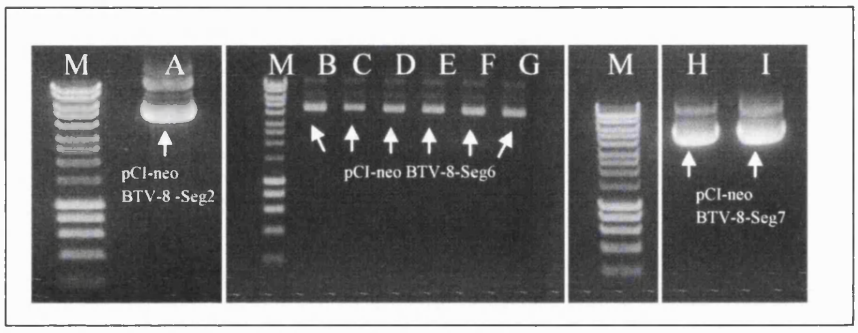


Figure 6-4 plasmid minipreps prepared as DNA vaccine candidates

Bacterial clones containing recombinant pCI-neo for minprep preparation (DNA vaccines), were tested by PCR and 1% AGE, using pCI-neo/F and pCI-neo/R primers, verifying the insertion of the correct sequence for BTV-8 Seg-2: BTV-8 Seg-6; BTV-8 Segt-7: Lane-M shows DNA marker (DNA Molecular Weight Marker (Hyperladder I); Lane A shows pCI-neo BTV-8-Seg2; Lanes B, C, D, E, F and G shows pCI-neo BTV-8-Seg6; Lanes H and I show pCI-neo BTV-8-Seg7.

6.2.2.5 : Detection of VP2, VP5 and VP7 specific mRNAs, in DNAse treated, RNA from recombinant pCI-neo transfected cells

In order to test the functionality of the BTV-specific expression cassettes of pCI-neo plasmids, total RNA (DNAse treated) was prepared from HEK293 cells transfected with: pCI-neo BTV-8-Seg-2; pCI-neo BTV-8-Seg-6; or pCI-neo BTV-8-Seg-7, then subjected to RT-PCR (using Transcriptor One-Step RT-PCR Roche kit) using BTV-gene specific primers (Chapter 2, section 2.8.5.3 table 2.14).

The absence of contaminating DNA in the RNA extracts, was confirmed by negative results for PCR (with no reverse transcription).using RNA templates pCI-neo BTV-8-Seg-2; pCI-neo BTV-8-Seg-6; from transfected HEK293 cells.

The products were analysed by 1% AGE, and amplicons were detected that were of the expected size (~2900 for Seg-2, ~1600 for Seg-6, and ~1200 bp for Seg-7 respectively) (Figure 6.5), confirming the presence of full length viral mRNAs in the samples. To rule out any possibility of amplification from the original plasmid inserts, the samples were also tested to confirm the absence of contaminating DNA in the RNA extracts by PCR, using the standard PCR reaction but without reverse transcription, giving

uniformly negative results. RNA template of pCI-neo BTV-8 Seg 2 and DNA pCI-neo BTV-8 Seg 6 but not with RNA template of pCI-neo BTV-8 Seg 7.

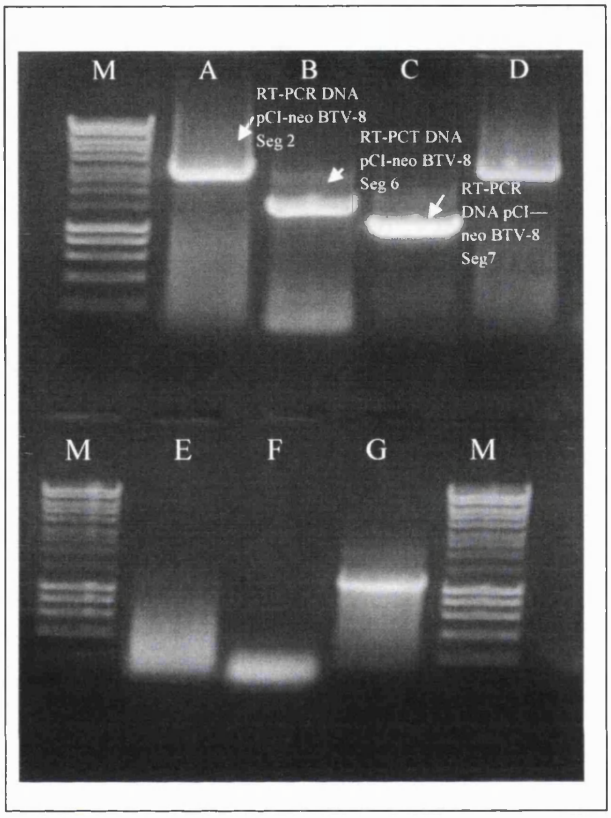


Figure 6-5 Detection of Seg-2, Seg-5 and Seg-7 specific mRNAs in cells transfected with pCI-neo BTV-8-Seg-2; pCI-neo BTV-8-Seg-6; or pCI-neo BTV-8-Seg-7

1% agarose gels, showing RT-PCR products from extracted RNA treated with DNAse, from HEK293 cells transfected with: Lane A, pCI-neo-BTV-8-Seg-2 (expected size 3000bp); Lane B, pCI-neo-BTV-8-Seg 6 (expected size 1600bp); Lane C pCI-neo-BTV-8-Seg-7 (expected size is 1200bp); ; Lanes E, F and G are standard PCR (no reverse transcription) products of transfected HEK293A cells with pCI-neo BTV-8-Seg-2; pCI-neo BTV-8-Seg-6; pCI-neo BTV-8-Seg-7 respectively (showing no products except for lane G This result could be due to the high concentration of DNA used in transfection which was not completely removed by the DNAse treatment using the RNeasy kit).Lanes M shows DNA Molecular Weight Marker (Hyperladder I).

6.3 : Generation of rMVA vaccines

The basic design for an rMVA vaccine-candidate, requires cloning of the target gene into a vaccinia virus transfer vector. The plasmid pSC11 (Chakrabarti et al., 1985), was used in this project. In this vector, foreign genes are inserted downstream of the vaccinia virus promoter P7.5. The lacZ gene is also present in this vector and is controlled by the Vaccinia promoter P11. This expression cassette (foreign gene and LacZ) is flanked by Vaccinia virus thymidine kinase gene DNA sequences, which enables the insertion of the expression cassette into the Vaccinia (or MVA) genome via homologous recombination. Recombinant viruses are selected by picking blue virus plaques upon staining of cells with Xgal.

In this project, cDNA copies of relevant ORFs (BTV-8 VP2, BTV-6 VP5 and BTV-8 VP7) were inserted into the pSC11 vector, under the control of Vaccinia virus promoter P7.5. DF-1 or CEF cells infected with vaccinia virus were transfected with the recombinant plasmids (pSC-11 BTV-8-Seg-2; pSC-11 BTV-8-Seg-6; or pSC-11 BTV-6-Seg-7) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) in order to generate the recombinant MVA-BTV viruses.

6.3.1 : Materials and Methods

To generate rMVA BTV-8VP2; rMVA BTV-8 VP5; and rMVA BTV-6 VP7, the open reading frame of each segment was amplified by PCR using primers containing SmaI cleavage sites. The resulting PCR products were digested with SmaI (New England Biolabs UK Ltd.) and inserted into the pSC11 at the SmaI site, downstream of the P7.5 promoter.

The plasmids that were generated were analysed by agarose gel electrophoresis and used to transfect CEF cells (as described in Chapter 2 section 2.23.5 a). The resulting clones were screened by sequencing using pSC-11 specific primers (table 2.8) to identify the correct insert and the orientation. Recombinant pSC11 plasmids containing appropriate inserts were used to generate recombinant viruses.

DF-1 cells infected with MVA at a 0.1 multiplicity of infection (moi) were transfected with the recombinant plasmid (pSC-11 BTV-8-Seg- 2; pSC-11 BTV-8-Seg- 6; or pSC-11 BTV-6-Seg- 7). The recombinant MVA viruses generated, were plaque selected through repeated rounds of plaque-picking of blue plaques (Chapter 2 section 2.23.6). The plaque purification process was repeated until only blue plaques were observed indicating the stock is free from wild type MVA.

RNA extraction and RT-PCR was carried out on the CEF infected with rMVA/BTV8VP2; rMVA/BTV8VP5; and rMVA/ BTV6VP7, using f/BTV8_VP2_vac, r/BTV8_VP2_vac, f/BTV8_VP5_vac, f/BTV8_VP5_vac, f/BTV6_VP7_vac and f/BTV6_VP7_vac primers (table 2.13). The expression level of VP2 and VP5 was tested by immunoblotting analysis using mouse polyclonal serum raised against BTV-8 VP2 and serum raised against BTV-8 (VP2complete+ VP5+ VP7) (prepared in house as described in Chapter 3) see figure 6.11. The expression level of VP7 was tested by immunoblotting analysis using mouse polyclonal serum raised against BTV-8 (VP2complete+ VP5+ VP7) (prepared in house as described in Chapter 3) However the signal was too weak to justify the correct expression of VP7 from rMVA/ BTV6VP7. Anti VP7 antibodies were however determined in mice vaccinated with VP2+VP5+VP7 in MVA-MVA prime-boost vaccination (Group 4 from table 2.17) see figure 6.12 indicating that rMVA/BTV6VP7 must have expressed VP7 in vivo which was identified by anti VP7 antibodies found in serum obtained from mice vaccinated with VP2+VP5+VP7 in DNA-MVA.

6.3.2 Results

6.3.2.1 : PCR amplification of Seg-2, Seg-6 and Seg-7 cDNAs for cloning into pSC11

BTV-8 Seg-2, Seg- 6 and BTV-6 Seg-7 were amplified from full length cDNAs using site specific primers (as shown in Chapter 2, Table 2.13) prior to their insertion into pSC11. All of the products were amplified successfully (Figure 6.6)

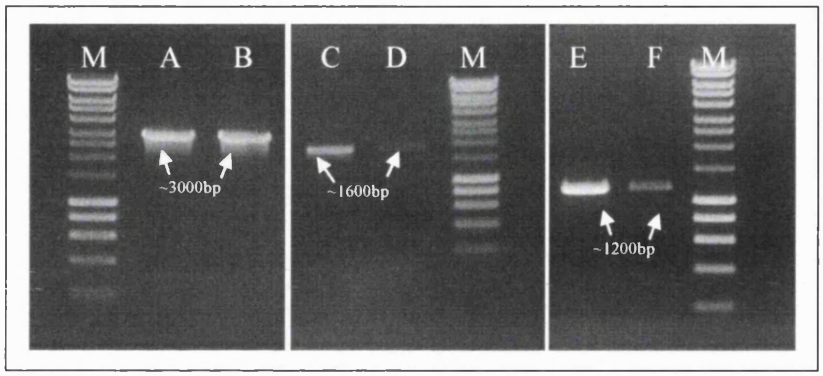


Figure 6-6 PCR products of BTV-8 Seg-2, Seg-6 and BTV-6 Seg-7 for insertion into pSC11:

BTV-8 Seg-2 & Seg-6 and BTV-6 Seg-7 were amplified from full length cDNA (as described in Chapter 2 section 2.8.2.1) using the site specific primers for cloning in Seg-2 into pSC11, and were analysed by 1% AGE: Lane-M shows DNA marker (Hyperladder I); Lane A and B: show cDNA of BTV-8 Seg-2; Lane C and D show cDNA of BTV-8 Seg-6; Lane E and F show cDNA of BTV-6 Seg-7. All primers are shown in Table 2.13

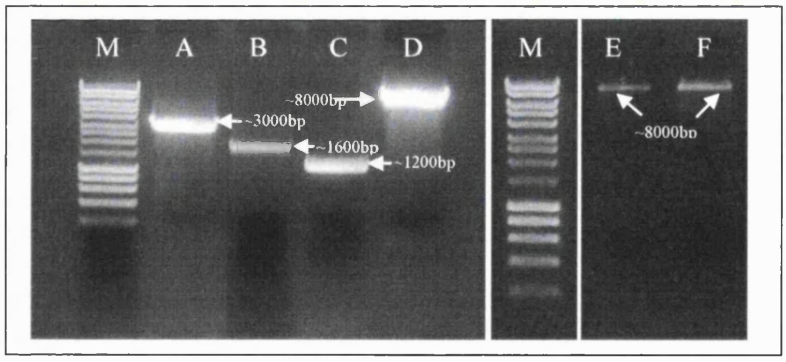


Figure 6-7 AGE analysis of SmaI digested cDNAs for cloning into pSC11:

PCR product from BTV-8 Seg-2 & Seg-6 and BTV-6 Seg-7 (generated as illustrated in figure 6.6) were digested with SmaI for cloning into pSC11. The digested products were analysed by 1% AGE. Lane-M shows DNA marker (DNA Molecular Weight Marker (Hyperladder I) Lane A: shows digested BTV-8 Seg-2 PCR products; Lane B shows digested BTV-8 Seg-6 PCR products; Lane C shows digested BTV-6 Seg-7 PCR products; Lane D shows SmaI digested pSC11; Lane E and F shows dephosphorylated pSC11.

6.3.2.2 : Digestion of pSC11 and BTV-8 Seg-2 & Seg-6 and BTV-6 Seg-7 cDNAs

The PCR amplified cDNAs of Seg-2, Seg-6 and Seg-7 were digested using SmaI (New England Biolabs UK Ltd. - as described in Chapter 2, section 2.8.6.2). The digested products were analysed by AGE (Figure 6.7).

6.3.2.3 : Colony screening of E. coli cultures for plasmid inserts using PCR

The digested PCR products from BTV-8 Seg-2 & Seg-6 and BTV-6 Seg-7 were cloned into pSC11. Bacterial colonies from each transformation were subjected to colony PCR testing (as described in Chapter 2 section 2.6.4) using pSC11/F and pSC11/R primers (Table 2.8) then analysed by 1% AGE (Figure 6.8).

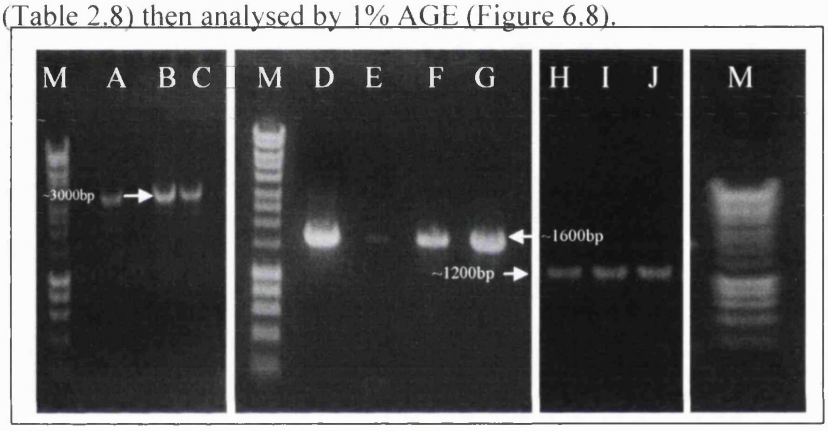
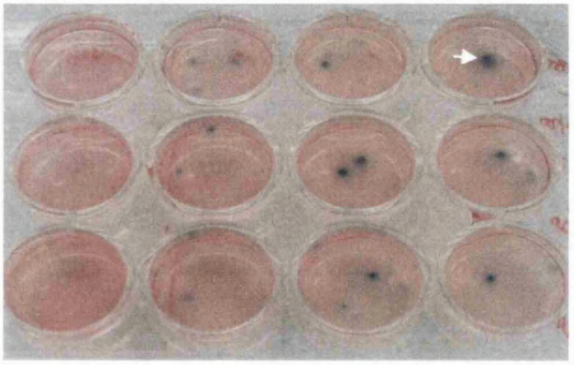


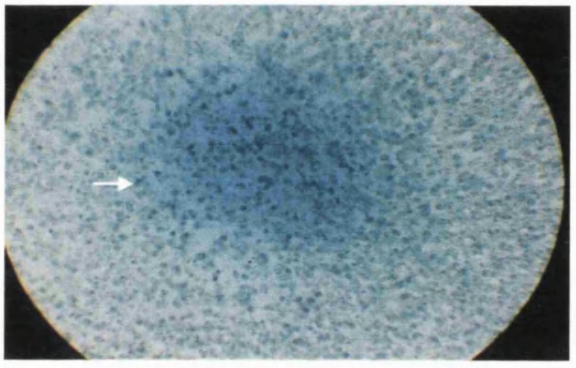
Figure 6-8 Cloning of cDNA in pSC11 vector and testing colonies by PCR:
Colonies obtained from transformation of XL1-Blue bacteria, were tested by PCR using pSC11/F and pSC11/R primers, and the products were analysed by 1% AGE: Lane-M shows DNA marker (DNA Molecular Weight Marker (Hyperladder I); Lanes A, B and C show pSC11 BTV-8-Seg-2, transformed into XL1-Blue cells; Lanes D, E, F and G show pSC11 BTV-8-Seg-6 transformed into XL1-Blue cells; Lane H, I, J show pSC11 BTV-6-Seg-7 transformed into XL1-Blue cells. pSC11/F and pSC11/R primers used for colony screening are shown in (Table 2.8).

6.3.2.4 : Selection of recombinant MVA viruses by plaque assay

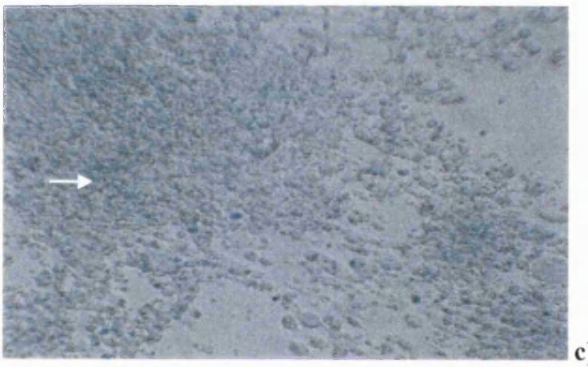
CEF or TK-BHK cells were infected with a 'transfection / co-infection harvest' (parental rMVA) or individual plaque picks representing rMVA BTV-8-VP2; rMVA BTV-8-VP5; or rMVA BTV-6-VP7, using various dilutions. Plates were incubated at 37°C for 1 hr. 1% LMP agarose was added following the removal of inocula and incubated for a further 2-3 days. Selection of recombinant MVA was done as described (Chapter2 section 2.23.6). Representative examples of the rMVA selection process are shown in Figure 6.9. The titre of the final rMVA virus stock was determined using β-Gal Staining (Invitrogen) (Figure 6.9).



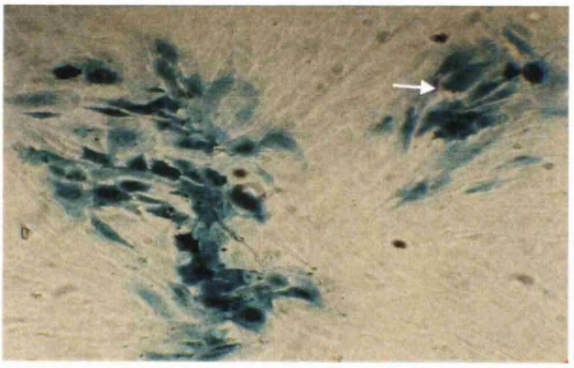
a)



b)



c)



d)

Figure 6-9 Selection of recombinant viruses (rMVA BTV-8 VP2 , rMVA BTV-8 VP5 or rMVA BTV-6 VP7) by Plaque assay

a) Visual selection of β-Galactosidase positive recombinant MVA

b) Microscopic selection of β- Galactosidase positive recombinant MVA in CEF

c) Microscopic selection of recombinant TK- phenotype) in TK-BHK infected cells

d) β-Gal Staining selection of recombinant MVA in CEF

6.3.2.5 : Detection of BTV-VP2, BTV-VP5 and BTV-VP7 cDNA from DNAse treated, total RNA of rMVA-VP2, rMVA-VP5 or rMVA-VP7 infected CEF cells

Total RNA (DNA-se treated) prepared from rMVA BTV-8-VP2, rMVA BTV-8-VP5 or rMVA BTV-6-VP7 infected CEF cells, were used in one step RT-PCR using primers shown in (table 2.13). The amplicons generated were of the expected size (~1600 for VP5, ~2900 for VP2 and ~1200 bp for VP7) (Fig.6.10). The absence of contaminating DNA in the RNA extracts, was confirmed by negative results for PCR (with no reverse transcription).using RNA templates rMVA-VP2; rMVA-VP5; rMVA-VP7 from infected CEF cells.

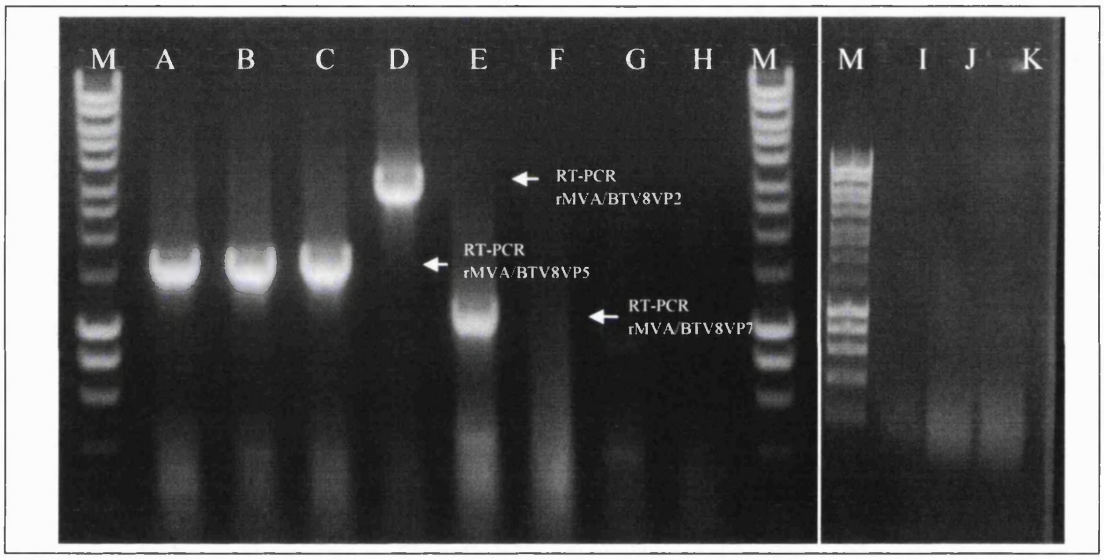


Figure 6-10 Detection of BTV-VP2, BTV-VP5 and BTV-VP7 specific DNA amplicons from DNAse treated, total RNA of rMVA-VP2, rMVA-VP5 or rMVA-VP7 infected CEF.

1% AGE analysis of RT-PCR products of RNA from CEF cells infected with rMVA BTV-8-VP5; rMVA BTV-8-VP2; rMVA BTV-6-VP7 respectively, using BTV specific primers (Table 2.13). Lane M shows (DNA Molecular Weight Marker (Hyperladder I). Lanes A to C show DNA amplicons from 3 different clones of rMVA BTV-8-VP5 (expected size - 1600bp); Clone C was used in the animal experiment; Lane D shows the DNA amplicon from rMVA BTV-8-VP2 (expected size - 3000bp); Lanes E, F and G show DNA products from rMVA BTV-6-VP7 (expected size - 1200bp), only one gave a positive signal (shown in lane E). Lane H is a –ve control, where RNA was extracted from CEF cells infected with wild MVA; Lanes I, J and K are standard PCR (no reverse transcription) products of rMVA-VP2, rMVA-VP5 or rMVA-VP7 infected CEF (showing no products).

6.3.2.6 : Confirmation of VP2 and VP5 expression from recombinant rMVA-VP2, rMVA-VP5 infected CEF cells by Western blotting

Expression of recombinant VP2 was confirmed by Western blotting using VP2 specific antibodies raised in mice (Chapter 5, Section 5.3.1.1). CEF cells infected with rMVA BTV-8VP2 were tested with mouse anti VP2 antiserum. A strong signal was detected, at ~116 KDa (the expected size of VP2), indicating that the rMVA strains expressed VP2 (Figure 6.11). Four different plaques were picked as described in (Chapter 2 section 2.23.6.2). The plaque pick that gave the greatest signal (plaque C) in figure 6.11 was used in the animal experiment. VP5 expression was confirmed by Western blotting using serum raised in mice against BTV-8 (VP2complete +VP5 + VP7) (prepared in house as described in Chapter 3) (see figure 6.11 lane E). VP7 expression was not detected directly from rMVA BTV-6 VP7 using Sheep and rabbit post infection serum (data not shown). Anti VP7 antibodies were however determined in mice vaccinated with VP2+VP5+VP7 in MVA-MVA prime-boost vaccination (Group 4 from table 2.17) see figure 6.12 lane A indicating that rMVA/BTV6VP7 must have expressed VP7 in vivo raising anti VP7 antibodies which were detected in serum obtained from mice vaccinated with VP2+VP5+VP7 in (DNA-MVA) prime boost vaccination.

Figure 6-11 Detection of VP2 (MVA expressed), using anti VP2 serum.
CEF cells infected with rMVA-BTV-8-VP2 or with rMVA-BTV-8-VP5 for 24 hours were analysed by immunoblotting (Chapter 2 section?) using anti-VP2 serum raised in mice inoculated with bacterial expressed VP2, or anti VP5 serum raised in mice inoculated with bacterial expressed VP2, VP5 and VP7 (as described in Chapter 5) respectively. Lane (M) shows protein marker (dual colour protein molecular weight marker (BioRad)) Lanes A, to D show protein expressed by rMVA-BTV-8VP2 identified by anti VP2 antibody (approximately 116KDa); Lane C shows the strongest signal, which was used in vaccination trial. Lane X is a negative control (CEF infected

with wild MVA immunoblotted with anti VP2 antibody); Lane E shows protein expressed by rMVA- BTV-8-VP5, identified by anti VP5 antibody (approximately 60KDa).

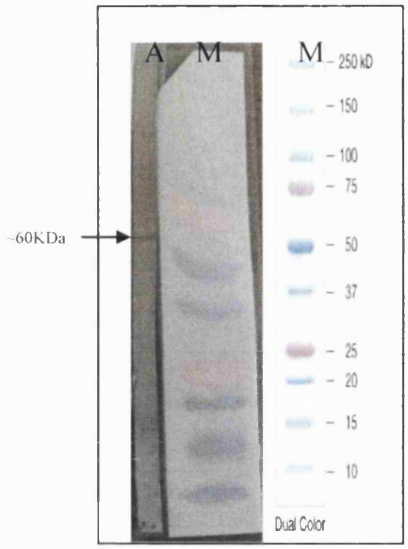


Figure 6-12 Detection of GST-VP7 with MVA-VP7 vaccinated mice antisera
Lane (M) shows protein marker (dual colour protein molecular weight marker (BioRad)) Lanes A shows expression of VP7 following vaccination with DNA-MVA (Group 4) vaccinated with (VP2+ VP5 + VP7), cellulose transfer membrane treated with VP7 expressed in bacteria as (described in chapter 2 section 2.11.1) was used to detect anti VP7 antibodies following vaccination with DNA-MVA expressing (VP2, VP5 and VP7) (Group 4) see (table 2.17). The signal was of the expected size of (38KDa VP7+ 26KDa of GST tag) giving an approximate size of ~64KDa.

6.4 Conclusion

One of the objectives of this project was to test differences in immune response and protection, following vaccination with DNA and or MVA expressing proteins *in situ*. This chapter describes the successful generation of DNA and rMVA vaccine candidates expressing BTV-8 VP2, BTV-8 VP5 and BTV-6 VP7 genes. These constructs were shown to contain transcriptionally active genes. In most cases the proteins of interest were expressed from these rMVA and DNA plasmid vectors. These were used for vaccination experiments in Chapter 7

For preparation of DNA vaccines, cDNA copies of relevant ORFs were inserted into the pCI-neo Mammalian Expression Vector, under the human cytomegalovirus (CMV) immediate-early enhancer/promoter region (pCI-neo, Promega). The CMV enhancer/promoter, promotes constitutive expression of cloned DNA inserts in mammalian cells. For preparation of rMVA vaccines, cDNA copies of relevant ORFs were inserted into the pSC11 transfer vector under the control of the 7.5 promoter.

Seg-2 encoding VP2 was cloned successfully in pCI-neo and pSC11 for use as a DNA and for rMVA preparation respectively. Although, generic primers were used for screening purposes they were unable to amplify the complete segment. Primers were therefore designed to amplify BTV genes, as described in Chapter 2, which proved valuable in detecting amplifying and sequencing the complete segment ORF.

Although Seg-6 encoding VP5 is much smaller than Seg-2, difficulties were encountered in cloning, possibly as a result of leaky expression. Cloning was only possible when the transformed vectors were plated and incubated at room temperature for 3-4 days, as incubation at 37°C failed to generate any colonies with the correct insert. Seg-7 of BTV-8 and BTV-6 were used for the preparation of DNA and rMVA respectively, no problems were encountered during cloning or expression of VP7, possibly (partly) because of its smaller size.

DNA amplicons were successfully generated from RNA transcripts of pCI-neo-BTV-8-Seg-2; pCI-neo-BTV-8-Seg-6; rMVA-BTV-8-VP2; rMVA-BTV-8-VP5; pCI-neo-BTV-8-Seg-7; or rMVA-BTV-6-VP7. The extracted RNA were all negative by PCR (if the RT step was omitted) confirming RNA as the effective template (Figures 6.5 and 6.10). Seg-7 was the only one that showed a positive signal by PCR on RNA templates (Figure 6.5). This result could be due to the high concentration of DNA used in transfection which was not completely removed by the DNAse treatment using the RNeasy kit.

The expression of viral proteins by MVA was confirmed for both VP2 and VP5, by western blotting, using serum from mice vaccinated with VP2, or with the three major proteins VP2, VP5 and VP7 expressed in bacteria. However, it was not possible to demonstrate the expression of VP7 by western blot, due to a lack of specific anti VP7 antibodies. Hyperimmune serum raised in rabbits against BTV-6 was used, but only identified very faint bands by western blot (data not shown). However, expression of VP7 *in situ* following vaccination with rMVA expressing VP7 was confirmed, as shown in Figure 6.12. The size of the band at approximately 60KDA was for VP7 as a GST fusion protein. Confirmation that VP7 was expressed in mice vaccinated with MVA expressing VP7 was provided by detection of antiVP7 antibodies.

Confirmation of proteins expression from DNA vaccines was not possible, due to the high level of expression required and the lack of sufficient suitable serum to test expression. In future a good antibody is needed to detect protein expression; this was not possible due the lack of facilities to conduct animal experiments at the Institute for animal health throughout the term of this project.

7 CHAPTER 7
Homologous (rMVA- rMVA) and heterologous (DNA-rMVA) prime boost vaccination of IFNAR (−/−) mice

Chapter 7: Homologous (rMVA- rMVA) and heterologous (DNA-rMVA) prime boost vaccination of IFNAR (−/−) mice

7.1 Introduction

Recombinant live viruses expressing foreign antigens have been used widely as vaccine delivery systems for infectious diseases and cancer (Wang et.al 2010). Although there are several recombinant vectors, earlier studies show that antigen production of human immunodeficiency virus genes by recombinant MVA was greater than that of canarypox virus vector due to longer duration of antigen production in recombinant MVA infected cells when compared to that of a canarypox virus vector (Zhang et al., 2007). MVA is one of the most promising vaccine vectors which has a well established safety record and history of use as a vaccine for infectious diseases and malignancies (Esteban 2009, Kennedy and Greenberg 2009)

There is some controversy regarding the efficacy of repeated administration of MVA vaccines. Some authors claim that the presence of circulating virus-specific neutralising antibodies might reduce the effectiveness of homologous boosting (Moore and Hill 2004). However, (Ramiraz et.al., 2000), stated that repeated inoculation with MVA is possible due to the low level of circulating anti vaccinia virus IgG antibodies in MVA inoculated mice. This is believed to be because MVA does not multiply productively. Therefore, the low neutralising antibodies against the vector may increase the antibody response to the foreign antigen.

Recombinant vaccinia virus (MVA) has been used successfully to express BTV proteins. DNA immunisation on the other hand represents a novel approach to vaccination and immunotherapeutic development. However, DNA vaccines have failed to provide high levels of protection when used alone, as compared to the protection induced following a DNA prime - MVA boost vaccination, which induced 100% protection from malaria (Moore and Hill 2004). Similarly a DNA-MVA vaccination strategy was more immunogenic than MVA-MVA homologous prime boost vaccination as heterologous vaccinations is believed to induce higher CTLs compared to each administrated separately.

Although many reports indicate the success of DNA-MVA prime boost vaccination (Hanke et.al., 1998; Schneider et al., 1998; Abaitua et.al., 2006), including BTV (Calvo-Pinilla et al., 2009b) however, vaccination with a closely related arbovirus (AHSV Serotype-4) VP2, using an rMVA-rMVA prime boost strategy has also provided

complete protection against a homologous virus challenge (Castillo-Olivares et al. 2011).

The establishment of a murine model for BTV-8 infection, (Calvo-Pinilla et al., 2009) has facilitated efficiency testing of BTV vaccination strategies. Published data suggest that a combination of the three major BTV proteins VP2, VP5 and VP7, gives better protection than VP2 and VP5, or VP2 alone, (Roy et al., 1990; Calvo-Pinilla et.al., 2009a). Indeed, co-expression (by recombinant baculovirus) of the four major BTV structural proteins (VP2, VP3, VP5 and VP7) can result in their assembly into 'virus like particles' (VLP) that raise both neutralising antibodies and a protective response in sheep (Roy P, 1992). However, studies with BTV and other related orbiviruses indicate that complete protection can be achieved by sub-unit vaccines based on the VP2 protein alone (Scanlen et al., 2002, Stone-Marschat et al.,1996). An MVA based vaccine expressing VP2 of African horse sickness virus AHSV Serotype 4 also provided complete protection in IFNAR (-/-) mice against homologous AHSV-4 challenge (Castillo-Olivares et al. 2011).

This chapter compares the protection efficacy of heterologous DNA/rMVA prime-boost and rMVA/ rMVA vaccination strategies. In addition, vaccination strategies based on BTV-8 VP2 (as sole antigen) or in a combination of VP2, VP5 and VP7, were also compared. These studies were conducted in the IFNAR (−/−) mouse model for BTV developed by Calvo-Pinilla et al. (2009).

7.2 : Materials and Methods

Two vaccination strategies were evaluated: either by priming with plasmid DNA containing cDNA copies of the BTV capsid-genes expressing protein VP2 alone, VP7 alone ; or VP2, VP5 and VP7, followed by a boost vaccination with recombinant Modified Vaccinia Ankara (rMVA) expressing the same proteins; or a prime-boost regime using rMVA (expressing these proteins) on both occasions. The DNA-rMVA, or rMVA-rMVA prime-boost – were administered at a three week interval and all of the animals were test bled on set dates throughout the experiment (Table 2.18). The vaccinated and un-vaccinated-control mice were subsequently challenged with a lethal dose (10pfu) of BTV-8 (Belgium/06 isolate). Clinical signs and survival rates were recorded. Virus neutralisation tests were used to assess neutralising antibody production in the vaccinated and / or challenged mice. The level of viraemia post-challenge was also determined by RT-qPCR and virus titration (Table 7.3)

7.3 : Results

7.3.1 : Post-challenge clinical signs

The clinical signs in vaccinated and unvaccinated-control mice were monitored for 13 days post challenge (pc), scored and recorded using a morbidity scoring system (see Chapter 2 section 2.18). All animals were observed and handled to evaluate clinical signs and animals with a score of 6 or more were humanely euthanized (Table 7.1 and 7.2).

Mice in group 1and 2 respectively were vaccinated with MVA-MVA and, DNA-MVA (respectively) expressing VP2, showed no clinical signs post challenge with a lethal dose of BTV-8. All mice in these groups survived till the end of the experiment. Group3 was vaccinated with DNA-MVA expressing VP7, started showing clinical signs on day 4 post challenge. All mice in this group either died or were humanely euthanized by day 7 post challenge.

Group1, 2, 4 and 5 vaccinated with heterologous DNA/rMVA prime-boost; or an rMVA/ rMVA vaccine strategy, for *in situ* expression of either BTV-8 VP2 (as sole antigen); or a combination of VP2/VP5 and VP7, showed no clinical signs post lethal BTV-8 challenge (Table 7.1). This indicates that expression of these proteins *in situ* protected the mice from clinical signs of infection. However, Group 3 (vaccinated with heterologous DNA/rMVA expressing VP7 alone) and Group 6 (unvaccinated control) showed severe clinical signs (Table 7.2) indicating that vaccination with VP7 alone was not protective. However, it was observed that in the VP7 vaccinated mice, the onset of clinical signs was delayed (Wilcoxon test: P=0.01) in comparison with the control group.

<table>
<tr><th>Groups</th><th>Animals</th><th>Days 1 to 4</th><th>Day 5</th><th>Day 6</th><th>Day 7</th><th>Days 8 to 13</th></tr>
<tr><td>Group 1 (MVA-MVA: VP2)</td><td>1.1 to 1.6</td><td colspan="5">No CS</td></tr>
<tr><td>Group 2 (DNA-MVA: VP2)</td><td>2.1 to 2.6</td><td colspan="5">No CS</td></tr>
<tr><td rowspan="5">Group 6 (DNA – MVA: VP7)</td><td>3.1</td><td rowspan="5">No CS</td><td>2 points(face and body fur ruffled), + 2points(Huddled and inactive) Total = 4</td><td colspan="3" rowspan="3">Dead</td></tr>
<tr><td>3.2</td><td>2 points (face and body fur ruffled) + 2 points (Huddled and inactive) +3 points (loss of fat and muscle, bones prominent), Total = 7. This mouse was humanely killed</td></tr>
<tr><td>3.3</td><td>2 points (face and body fur ruffled) + 2 points (Huddled and inactive) +1 points (thin) Total = 5</td></tr>
<tr><td>3.4</td><td>2 points (face and body fur ruffled) + 2 points Huddled and inactive) Total = 4</td><td>*</td><td>2 (points face and body fur ruffled) + 2 points Huddled and inactive) +1 points (thin) Total = 5 This animal died later that day</td><td rowspan="2">Dead</td></tr>
<tr><td>3.5</td><td>NoCS</td><td>*</td><td>2 points (face and body fur ruffled) + 2 points (Huddled and inactive) +1 point (lacrimation in one or both eyes), Total =5 This animal died later that day</td></tr>
</table>

Table 7-1 Post-challenge clinical signs in Group 1, 2 and 3.
Mice were vaccinated with (MVA-MVA), (DNA-MVA) expressing VP2 *in situ* and challenged with a lethal dose of BTV-8, showed no clinical signs post challenge and all mice in these groups survived till the end of the experiment. Group 3 as vaccinated with (DNA-MVA) expressing VP7 *in situ* and challenged with a lethal dose of BTV-8, started showing clinical signs on day 4 post challenge and all mice in this group either died or were humanely euthanized by day 7 post challenge. **No CS = No clinical signs * = animal not examined**

Groups	Animals	Days 1 & 2	Day 3	Day 4	Day 5	Days 6 to 13
Group 4 (cDNA-MVA: VP2+VP5+VP7)	4.1 to 4.6	No CS				
Group 5 (MVA-MVA: VP2+VP5+VP7)	5.1 to 5.6	No CS				
Group 6 Unvaccinated control	6.1	No CS	No CS	2 points (face and body fur ruffled) + 2 points (loss of fat no growth) +1 point (apathy isolated but responsive to noise) Total = 5 points	3 points (lack of grooming, unkempt thin coat) +3 points (loss of fat and muscle, bones prominent), Total = 6 This mouse was humanely killed	Dead
	6.2			2 points (face and body fur ruffled), Total = 2 points	3 points (lack of grooming, unkempt thin coat) +3 points (loss of fat and muscle, bones prominent) Total = 6 This mouse was humanely killed	
	6.3		2 points (face and body fur ruffled) +1 points (apathy isolated but responsive to noise) Total = 3 points	2 points (face and body fur ruffled) +3 points (conjunctivitis, crusting, unable to open eyes) Total = 5 points	3 points (lack of grooming, unkempt thin coat) +3 points (loss of fat and muscle, bones prominent) +3 points (conjunctivitis, crusting, unable to open eyes) Total = 9 This mouse was humanely killed	
	6.4		No CS	2 points (face and body fur ruffled) Total = 2 points	3 points (lack of grooming, unkempt thin coat) +2 points (huddled inactive) +1 points (thin) Total = 6 This mouse was humanely killed	
	6.5			2 points (face and body fur ruffled) +1 point (apathy isolated but responsive to noise) Total = 3 points This mouse was humanely killed	3 points (lack of grooming, unkempt thin coat) +3 points (loss of fat and muscle, bones prominent), Total = 6 This mouse was humanely killed	

Table 7-2 Post-challenge clinical signs (CS) in Group 4,5 and 6

Mice in group 4 and 5 respectively were vaccinated with (DNA-MVA), (MVA-MVA) expressing VP2, VP5 and VP7 in situ and challenged with a lethal dose of BTV-8 showed no clinical signs post challenge and all mice in these groups survived till the end of the experiment. Group6 (control), were challenged with a lethal dose of BTV-8 and started showing clinical signs on day 3 post challenge and all mice in this group were humanely euthanized by day 5 post challenge. **No c. s = No clinical signs * = animal not examined**

7.3.2 : Post challenge survival rates in mice vaccinated with heterologous (DNA-rMVA) and homologous (rMVA- rMVA) prime boost vaccination

The efficacy of heterologous DNA-MVA and homologous MVA-MVA prime boost vaccination strategies for protection against BTV, were tested in IFNAR -/- mice. The mice were inoculated with cDNAs and / or rMVAs, expressing VP2 alone, VP7 alone or VP2, VP5 and VP7, as shown in (Table 2.17). The mice were challenged 14 days after boosting, with a lethal dose (10PFU) of BTV-8, by a sub-cutaneous route. All mice in group 6 (non-vaccinated control-group) showed clinical signs of BTV infection with scores reaching a minimum of 6 on day 5 pi. Therefore, all mice in group 6 were euthanized. Two mice in group 3 (mouse number 3.2 and 3.6) vaccinated with (DNA+MVA - VP7), showed sever clinical signs and were 'sacrificed' on day 5 pi. The remaining four mice in this group died by day 7 pi. Mice vaccinated with heterologous DNA-MVA or homologous MVA-MVA prime-boost regimes based on VP2 alone (groups 1 and 2), survived the challenge with no clinical signs of infection. One mouse in group 5 (died on day 7 pi), with no obvious clinical signs of disease. However, all of the remaining mice vaccinated with VP2, VP5 and VP7, as DNA-MVA (group 5) or MVA-MVA (group 4), survived challenge and showed no clinical signs until the end of the experiment (day13 pi) (Figure 7.1).

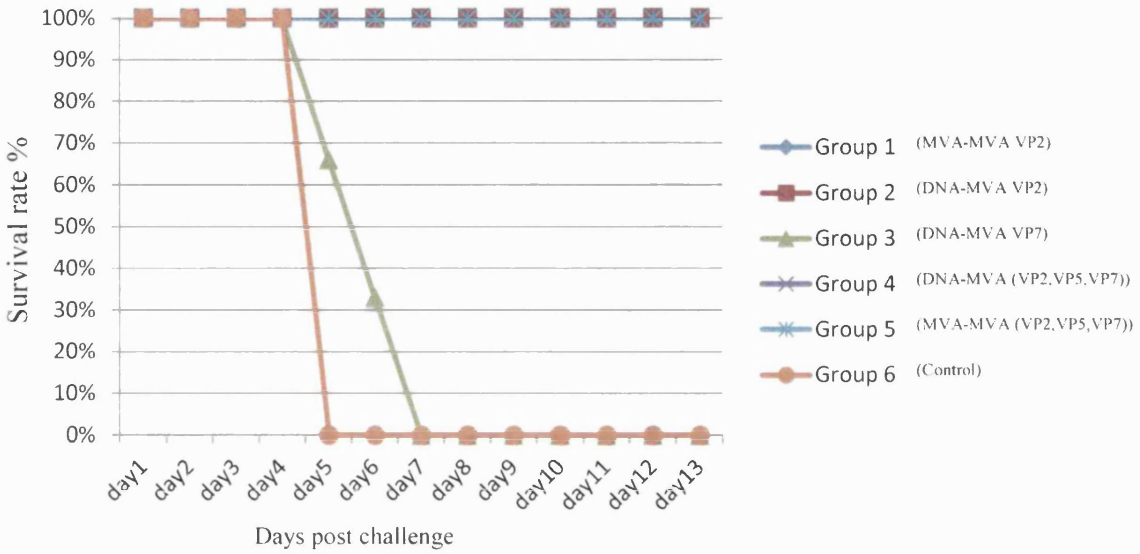


Figure 7-1 In vivo protection of IFNAR -/- mice vaccinated with VP2 VP5 and VP7, from BTV-8 challenge

Five groups of mice (each consisting of 6 mice), were vaccinated twice at 3 weeks interval. Group 1 was vaccinated with homologous prime/boost vaccination (rMVA-rMVA) expressing VP2. Group 2 was vaccinated with heterologous prime boost vaccination (DNA-rMVA) expressing VP2. Group 3 was vaccinated with heterologous prime boost vaccination (DNA-rMVA) expressing VP7. Group 4 was vaccinated with heterologous prime boost vaccination (DNA-rMVA) expressing VP2, VP5 and VP7. Group 5 was vaccinated with heterologous prime boost vaccination (rMVA –rMVA) expressing VP2, VP5 and VP7. A sixth group (untreated control) was challenged, along with the remaining groups, on day 34 of the experiment (2 weeks post boost in the vaccinated groups). Clinical signs were recorded daily and survival rates were determined for 13 days post challenge. Groups 1, 2, 4 and 5 were completely protected, showing no clinical signs and survived till the end of the experiment (13 days post challenge). In group 3 two mice were euthanized on day 5 post challenge and the remaining mice died by day 7 post challenge. Group 6 showed severe clinical signs and were humanely euthanized on day 5 pi.

7.3.3 : Neutralising antibodies in vaccinated mice

Blood samples were collected from all experimental mice on days 0, 20 and 34 post vaccination (pv), and on day 7 and 13 pi. Virus neutralising antibodies were not detected in any of the groups of mice following the prime vaccination (day 20) (Figure 7.2). However, Low levels of neutralising antibodies were detected against BTV-8 on day 34 (two weeks post boost) in all mice that had received VP2 expressed from DNA + rMVA, or rMVA + rMVA (either alone or in combination with other BTV proteins). Titres on day 34 pi did not differ significantly ($P>0.05$) amongst these groups. These antibodies reached a maximum mean titre of 1.15 in Group 5 prior to challenge (Figure 7.2), increasing again on day 7 and day13 pi, to reach a maximum titre >3. No

neutralising antibodies were detected in serum from mice vaccinated with VP7 alone, or in serum from the control group (group 6). This may in part reflect the rapid death of these animals by day 7 or day 10 pi (Table 2).

The level of neutralising antibodies post challenge was significantly ($P<0.02$) higher in group 5 compared with groups 1, 2 and 4 (Figure7.2), possibly due to a further 'boost' caused by the partial replication of the challenge viruses (tables 7.3 and 7.4). In contrast groups 1, 2 and 4, which developed slightly higher neutralising antibodies post vaccination, were more effectively protected, with lower levels of challenge-virus replication (tables 7.3 and 7.4), and developed lower neutralising antibody levels by day 13 pi (Figure 7.2).

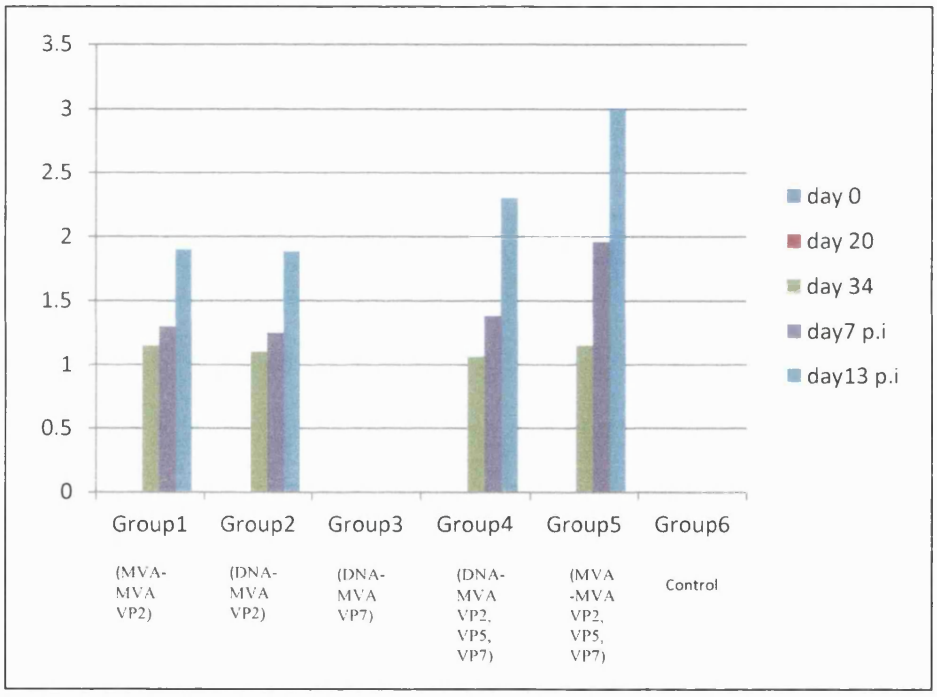


Figure 7-2 Production of neutralising antibodies against BTV-8 in IFNAR -/- mice vaccinated with heterologous (DNA-rMVA) and homologous (rMVA- rMVA) prime boost vaccination

Virus neutralisation tests were carried out on serum samples taken from vaccinated and control mice on day 0, day 20, day 34 post vaccination (pv), and on day 7 and 13 (pi). Groups 1, 2, 4 and 5 vaccinated with (rMVA-rMVA VP2); (DNA-rMVA VP2); (DNA-rMVA (VP2, VP5, VP7) and (rMVA-rMVA (VP2, VP5, VP7) respectively, showed significant levels of neutralising antibodies following boost vaccination. This level increased still further on 7 and day13 pi. No neutralising antibodies were detected in Groups 1, 2, 3, 4 and 5 following prime vaccination. No neutralising antibodies were detected in Group 3 post boost vaccination, or in the control group (Group 6). Mice in these groups were dead by day 7 pi.

7.3.4 : Viraemia in mice after challenge with BTV-8

The level of viraemia in challenged mice was measured by plaque assays on days 3, 5, 7, 10 and 12 pi. No virus was detected in any of the blood samples taken on day 3 pi. However, all of the mice in groups 3 and 6 (vaccinated with VP7, or unvaccinated controls, respectively) developed viraemia > $1x10^3$ PFU/ml prior to death (table 7.4).

Three mice in group 4 and two mice in group 5 (both groups vaccinated with VP2, VP5 and VP7, which all survived) also developed viraemia, albeit at significantly ($P<0.02$) lower titres than in groups 3 and 6 ($\leq$ 3x102 PFU/ml). No viraemia was detected in any of the mice in groups 1 and 2 (vaccinated with VP2 only).

7.3.4.1 . Detection of BTV-8 RNA by qRT-PCR in blood samples from a mouse vaccination / challenge study

Whole blood samples collected from vaccinated and challenged mice were used to detect BTV RNA by RT-qPCR. (Table 7.3) Four of the unvaccinated control mice were positive on day 3 (Ct values of 30.3 to 34.56) and all six animals were positive on day 5 pi (Ct values of 24.07 to 29.09). Three mice in group 3 (vaccinated with VP7 alone) also had low levels of BTV RNA in their blood on day 3 (Ct of 31.79 to 35.15), which had increased by day 5pi (Ct of 23.85 to 26.34). The three remaining mice in group 3 were also positive on day 5 (Ct of 30.67 to 32.7).

In groups 4 and 5 which were vaccinated with (DNA-MVA - VP2, VP5, VP7; or rMVA-rMVA -VP2, VP5, VP7 respectively), BTV RNA was detected throughout the experiment, although not in all animals (Table 7.3). In (group 1) vaccinated with VP2 (rMVA-rMVA), BTV RNA was detected in 5 mice but only on day 7 pi (Ct of 33.72 to 34.7), while in group 2 vaccinated with VP2 (DNA-rMVA) BTV RNA was only detected in a single mouse on days 5 and 7pi (Ct of 33.24 and 32.95) (Table 7.3).

Although all of the mice in groups 1, 2, 4 and 5 were protected from infection with BTV-8, showing no clinical signs and surviving till the end of the experiment), only vaccination with VP2 provided sterile protection in group 1 (rMVA-rMVA) and group 2 (DNA-rMVA) (Table 7.4).

		Days post challenge				
Groups	**Animals**	**day 3**	**day 5**	**day 7**	**day 10**	**day 12**
	1.1	no Ct.	no Ct.	34.7	No Ct	No Ct
	1.2	no Ct.	no Ct.	33.72	No Ct	No Ct
Group 1	1.3	no Ct.	no Ct.	34.24	No Ct	No Ct
(MVA-MVA VP2)	1.4	no Ct.	no Ct.	no Ct.	No Ct	No Ct
	1.5	no Ct.	no Ct.	33.93	No Ct	No Ct
	1.6	no Ct.	no Ct.	34.3	No Ct	No Ct
	2.1	no Ct.	no Ct.	no Ct.	No Ct	No Ct
	2.2	no Ct.	no Ct.	no Ct.	No Ct	No Ct
Group 2	2.3	no Ct.	33.24	32.95	No Ct	No Ct
(DNA-MVA VP2)	2.4	no Ct.	no Ct.	no Ct.	No Ct	No Ct
	2.5	no Ct.	no Ct.	no Ct.	No Ct	No Ct
	2.6	no Ct.	no Ct.	no Ct.	No Ct	No Ct
	3.1	35.15	26.34	Dead	Dead	Dead
	3.2	33.86	24.67	Dead	Dead	Dead
Group 3	3.3	no Ct.	30.67	Dead	Dead	Dead
(DNA-MVA VP7)	3.4	no Ct.	32.33	25.6	Dead	Dead
	3.5	no Ct.	32.7	31.2	Dead	Dead
	3.6	31.79	23.85	Dead	Dead	Dead
	4.1	no Ct.	34.9	31.55	31.37	32.8
	4.2	40.35	32.51	30.37	34.62	33.64
Group 4	4.3	33.91	31.47	29.96	No Ct	No Ct
(DNA-MVA-	4.4	34.2	31.55	30.7	31.97	No Ct
VP2,VP5,VP7)	4.5	31.9	31.47	31.9	31.33	33.62
	4.6	No Ct	33.65	35.07	No Ct	34.2
	5.1	No Ct	30.42	31.16	No Ct	No Ct
	5.2	No Ct	31.1	31.32	32.57	33.03
Group 5	5.3	No Ct	35.04	33.84	32.44	No Ct
(MVA-MVA-	5.4	34.13	42.06	32.61	30.85	34.77
VP2,VP5,VP7)	5.5	33.85	No Ct	34.49	No Ct	No Ct
	5.6	34.39	30.47	?????	Dead	Dead
	6.1	30.34	25.94	Dead	Dead	Dead
	6.2	32.4	24.82	Dead	Dead	Dead
Group 6	6.3	31.38	24.07	Dead	Dead	Dead
(Control)	6.4	34.56	28.02	Dead	Dead	Dead
	6.5	No Ct	29.09	Dead	Dead	Dead
	6.6	33.87	26.46	Dead	Dead	Dead

Results are expressed as Ct values.

Table 7-3Detection of BTV-8 RNA by qRT-PCR in blood samples from vaccinated and control mice at post challenge

BTV RNA was detected in mouse blood post challenge, by RT-qPCR, as early as three days post challenge. Day five was the last day blood samples were recovered from the control group (Group3) which had the highest Ct value (Mean 26.4).

7.3.4.2 : Viraemia in blood samples recovered from vaccinated and control mice post challenge

The level of viraemia in challenged mice was measured by plaque assay on days 3, 5, 7, 10 and 12 pi (as described in Chapter 2 Section 2.23.8). Table 7.4 shows that no virus was detected in any blood samples taken on day 3 pi. However, all of the mice in groups 3 and 6 (vaccinated with BTV-8 VP7, or unvaccinated controls, respectively), developed viraemia on day five post challenge (table 7.4). No viraemia was detected in mice vaccinated with rMVA-MVA VP2; or DNA-MVA VP2 post challenge. Group 4 and 5, vaccinated with DNA-MVA expressing VP2, VP5, VP7; or rMVA-MVA expressing VP2, VP5, VP7 (respectively) had a maximum level of $2x10^2$ pfu/ml on day 7 post challenge. All mice with virus titre of $\geq 10^3$ either died or were humanely euthanized.

Groups	Animals	Days post challenge day 3	day 5	day 7	day 10	day 12
	1.1	neg.	neg.	neg.	neg.	neg.
	1.2	neg.	neg.	neg.	neg.	neg.
Group 1	1.3	neg.	neg.	neg.	neg.	neg.
(MVA-MVA VP2)	1.4	neg.	neg.	neg.	neg.	neg.
	1.5	neg.	neg.	neg.	neg.	neg.
	1.6	neg.	neg.	neg.	neg.	neg.
	2.1	neg.	neg.	neg.	neg.	neg.
	2.2	neg.	neg.	neg.	neg.	neg.
Group 2	2.3	neg.	neg.	neg.	neg.	neg.
(DNA-MVA VP2)	2.4	neg.	neg.	neg.	neg.	neg.
	2.5	neg.	neg.	neg.	neg.	neg.
	2.6	neg.	neg.	neg.	neg.	neg.
	3.1	neg.	$2x10^3$	dead	dead	Dead
	3.2	neg.	$1.5x10^4$	dead	dead	Dead
Group 3	3.3	neg.	$2.5x10^3$	dead	dead	Dead
(DNA-MVA VP7)	3.4	neg.	neg.	$5.2x10^3$	dead	Dead
	3.5	neg.	$1x10^3$	$2.5x10^3$	dead	Dead
	3.6	neg.	$3x10^3$	dead	dead	Dead
	4.1	neg.	neg.	$1x10^2$	neg.	neg.
	4.2	neg.	neg.	neg.	neg.	neg.
Group 4	4.3	neg.	neg.	$1x10^2$	neg.	neg.
(DNA-MVA	4.4	neg.	neg.	$2x10^2$	neg.	neg.
VP2,VP5,VP7)	4.5	neg.	$1x10^2$	$2x10^2$	neg.	neg.
	4.6	neg.	neg.	neg.	neg.	neg.
	5.1	neg.	neg.	neg.	neg.	neg.
	5.2	neg.	$3x10^2$	neg.	neg.	neg.
Group 5	5.3	neg.	neg.	neg.	neg.	neg.
(MVA-MVA	5.4	neg.	$2x10^2$	neg.	neg.	neg.
VP2,VP5,VP7)	5.5	neg.	neg.	neg.	neg.	neg.
	5.6	neg.	neg.	dead	dead	Dead
	6.1	neg.	$6x10^3$	dead	dead	dead
	6.2	neg.	$5x10^3$	dead	dead	dead
Group 6	6.3	neg.	$1.2x10^4$	dead	dead	dead
(Control)	6.4	neg.	$1.5x10^3$	dead	dead	dead
	6.5	neg.	$6x10^3$	dead	dead	dead
	6.6	neg.	$2.5x10^3$	dead	dead	dead

Table 7-4 Detection of BTV-8 infectivity (viraemia) in mouse blood at various dates post challenge

The titre of BTV-8 in blood samples recovered from vaccinated (Group 1-5) and control -non vaccinated mice (Group 6) at various dates post challenge Results are expressed in PFU/ml values.

7.4 Conclusion

Both homologous (rMVA-rMVA) and heterologous (DNA-rMVA) prime boost vaccinations, expressing either VP2 alone or a combination of three major BTV proteins VP2, VP5 and VP7, all generated neutralising antibodies by two weeks post boost. The two groups of IFN (-/-) mice vaccinated with VP2 alone, using either DNA-rMVA or rMVA-rMVA strategies, were completely protected against clinical signs of BTV infection. They also and had no detectable viraemia, although a low level of BTV RNA was detected in some individuals by qRT-PCR.

Vaccination with VP2 of AHSV Serotype-4, using an rMVA-rMVA prime boost strategy was also previously shown also provide complete protection against a homologous virus challenge (Castillo-Olivares et al. 2011). These obsevations correlate with the known role of outer-capsid protein VP2 as the major 'neutralising antigen' of BTV and AHSV.

Earlier vaccination trials using DNA-rMVA expressing VP2, VP5 and VP7 of BTV-4, also protected IFN (-/-) mice from a lethal challenge (Calvo-Pinilla, E., et al., 2009). There is published evidence that VP5 can influence the determinants of BTV neutralisation (DeMaula et.al., 2000 Mertens et al., 1996). However, in the current study, co-expression of BTV-8 VP2, with VP5 and VP7 reduced the overall level of protection in mice. The earlier study with BTV-4 also reported that a combination of VP2 and VP5 failed to induce a protective response (Calvo-Pinilla, E., et al., 2009).

Although VP7 does not raise antibodies that can neutralise intact BTV particles, it can provide partial protection, possibly via a cell mediated immune response, and its incorporation is thought to enhance the efficacy of VP2 and VP5 vaccines (Wade-Evans et al., 1996; Roy et.al., 1990). However, in the current study, vaccination with DNA and / or rMVA expressing VP7 alone did not protect IFN (-/-) mice against BTV-8 challenge.

In order to prevent onward transmission of the virus, BTV vaccines should ideally generate sterile immunity, preventing virus replication and viraemia. Since clinical BT has never been reported in the absence of viraemia, this is likely to also protect vaccinated animals against clinical signs of the disease (Hoffmann et al., 2009).

Development of virus-vectored subunit-vaccines, allows the 'live' pathogen to be excluded, from the production process eliminating disease-security risks. These safe vaccines also prevent or avoid: the risk of reversion to virulence (e.g. by reassortment

with wild type strains); contamination with toxic compounds used for inactivation; any risk of incomplete inactivation of whole-cell vaccines. They are also compatible with DIVA surveillance strategies.

The results presented here show that VP2 expressed *in situ* using a heterologous or homologous prime boost vaccination (DNA-rMVA or rMVA-rMVA), can generate sterile immunity against BTV-8 in IFN -/- mice, protecting them against a lethal challenge. However, further work will be needed to be to test and validate the use and efficacy of these BTV-subunit vaccine candidates in ruminants (the natural hosts for BTV infection), including a further investigation of protection against heterologous serotypes.

8 CHAPTER 8
Final discussion and Future work

Chapter 8: Final discussion and Future work

8.1 : Introduction

The BTV-8 outbreak that started in northern Europe during 2006 has subsided, with no new cases recorded in the region since 2010. As a result the level of vaccination has also rapidly declined and had almost stopped in 2011. With rapid turnover in ruminant livestock (estimated at 20% per year), the northern European animal populations will rapidly return to naivety, increasing the risk of another outbreak caused by BTV-8, or possibly the introduction and emergence of yet another previously exotic type.

The outbreak in Europe caused by BTV-8, has been linked to climate change. It has been suggested that higher temperatures in the region, increased the geographic distribution of *Culicoides imicola* (an important BTV vector species), allowing to spread to more of southern Europe. High temperatures may also have increased the abundance and duration of adult vector activity, increasing vector capacity and the likelihood of continuous BTV transmission cycles (between adult vectors and hosts). Insects reared at higher temperatures also have an increased susceptibility to BTV infection and this may have helped to recruit novel vector species in northern Europe (Purse et al., 2005).

New strains of BTV have been generated in the Mediterranean region, through re-assortment between wild-type strains, as well as between wild type strains and the live vaccine viruses that have been used in several countries (Veronesi et al., 2005; Ferrari et al., 2005; Batten et al 2008). The epidemic of BTV-8 in 2006-2010, led to the manufacture of an inactivated BTV-8 vaccine and implementation of a voluntary vaccination programme in the UK, and subsequent compulsory vaccination programmes against BTV-8 in Germany and BTV-1 and 8 in France demonstrated the success of control strategies based on vaccination (Eschbaumer et al., 2009; Gethmann et al., 2009)

It is important that BTV vaccines are safe, while still inducing potent and long lasting immune responses. However it is also important that they have potential for the development of a reliable serological assay to 'distinguish infected from vaccinated animals' (DIVA assays). The work described in this thesis was therefore focussed on the study of two different approaches for developing new-generation subunit-vaccines for BTV, with DIVA capacity. Based on previous reports indicating the importance of VP2, VP5 and VP7 antigens (Roy et al., 1990; Wade-Evans et al., 1996 Calvo-Pinilla et.al., 2009b) for induction of protective immunity, these proteins, were chosen for further exploration of BTV vaccination strategies. The successful development of these

vaccines would allow non-structural antigens to be used for development of DIVA diagnostics (Barroe et al., 2009).

Although cytotoxic T lymphocyte responses play an important role in BTV immunity (Jeggo and Wardley, 1982a; 1982b; 1982c; 1985; Lobato et al 1997), virus neutralising antibodies are seen as an essential component of the adaptive immune response, for protection against BTV. They are therefore to be a good indicator of immunity against this disease. During my PhD I have evaluated the immune responses to some novel vaccine candidates using primarily serological methods (Serum Neutralisation Tests). However, additional protective cellular immune responses are likely to have been stimulated by these novel vaccination approaches, which may contribute to some level of cross-serotype protection.

Three strategies (sub-unit protein, plasmid DNA and MVA vectored vaccines) were used for the development of next-generation vaccine-candidates against BTV-8. The protection/immune responses that were generated were tested in a BT-mouse model (Calvo-Pinilla et al, 2009). Protective efficacy was assessed in vaccination / challenge trials, using a clinical scoring system, survival rates, virus-neutralising antibody titres and viraemia (qPCR and virus plaque assay).

8.2 : Bacterial expression of BTV structural protein (VP2, VP5 and VP7)

In order to develop a next-generation alternative to the inactivated or live-attenuated vaccines that are currently available, the major structural proteins (VP2, VP5 and VP7) were expressed (successfully) in a bacterial system, then evaluated as components of a subunit vaccine. Prokaryotic expression was chosen for its simplicity and cost-efficiency. Unlike mammalian or insect cell cultures, the culture and maintenance of bacteria does not require costly media and serum (Pathak et al., 2008). It is therefore a first choice for recombinant protein expression, and alternative systems should only be used after *E. coli* expression systems have been reasonably explored (Graslund et al., 2008).

VP2 is not only the most important BTV protein for stimulation of a neutralising antibody response (Maan et al 2007b); it also has a role in inducing cytotoxic T lymphocyte responses (Janardhana et a., 1999). Seg-2 (encoding VP2) was therefore targeted in initial work to express BTV proteins (VP2) for vaccinology studies. X-ray crystallography and high resolution structural studies indicate that solubility often increases if an expressed protein adopts a 'native' conformation. This 'conformation'

also promotes display of conformational epitopes that can potentially play an important role in protective immune responses (e.g. to VP2). However, Seg-2 is one of the largest BTV genome segments and in an attempt to obtain highly soluble expression products, the segment was constructed as three overlapping fragments, sharing approximately 370 bases (as shown in Appendix I).

The immuno-dominant sub-fragments of a immunogen, can be specifically selected and targeted to simplify production of subunit-vaccines (Liljeqvist, 1999), DeMaula et al., (2000) conclude that BTV neutralising epitopes are located at 199 - 213 (region 1) and at 321-346 (region 2) of VP2 in several BTV serotypes. However, no structural data is available concerning the position of these sites in the folded protein. The expression-constructs were therefore designed to cover the entire protein including these regions.

pGEX4T2 has a high copy number and is therefore recommended for expression purposes in both Prokaryotic and Eukaryotic systems. This vector allows fusion of a GST affinity tag to the N-terminal of the expressed protein. The GST tag can be used for purification and to aid folding and solubilisation of the expressed protein. N-terminal, fusion ensures that the bacterial transcription and translation machineries always encounter 5' and N- terminal sequence that are compatible with robust RNA synthesis and protein expression respectively (Graslund et al., 2008).

VP5 the second most variable BTV protein (Maan et al 2008) and is believed to enhance the generation of protective neutralisation activity of the antibodies raised to VP2 alone. Seg-6 encoding VP5 is only (1638) bp long and was not therefore expressed as multiple smaller fragments. Although, a cDNA construct of Seg-6 was cloned successfully in pGEX4T2, all attempts to express VP5 in bacteria were unsuccessful. This is perhaps not surprising, since earlier reports identified two amphi-pathic helices in the first 40 residues of the amino terminus of VP5, which are responsible for cytotoxicity (Hassan et al., 2001; Forzan et al., 2003). VP5 can act as a fusion protein inducing syncytium formation in a manner similar to the fusion proteins of enveloped viruses (Forzan et al., 2003). This could explain why attempts to express VP5 in *E. coli* were unsuccessful. Bearing in mind that recombinant protein toxicity could be host cell specific (Brondyk, W., 2009), different expression hosts were used (BL-21, C41 and C43) but were also unsuccessful. Although inducible promoters are desirable for production of toxic proteins (Makrides 1999), use of the tac promoter was unsuccessful for expression of VP5, which was still toxic the bacterial host.

Expression of membrane proteins is challenging and it has been suggested that production of the soluble, hydrophilic portion, can be facilitated by removal of the

membrane spanning domain (Brondyk, W., 2009). The hydrophobic regions are also responsible for interaction with the cell membrane. Their removal should therefore reduce the cytotoxic effect of the protein and allow the expression of a truncated version of the protein, containing relevant epitopes. This approach was attempted to allow expression of BTV-8 VP5, by removing the amphi-pathic helices.

Primers were designed for BTV-8 Seg-6 cDNA, starting 289 nt downstream from the start of the open reading frame. The resulting cDNA was successfully cloned into pGEX4T2 for expression of a truncated version of VP5 fused to a GST tag. This resulted in the expression of 50KDa of the VP5 (loss of almost ¼ of VP5 protein at the amino terminus) in E. coli. However, the truncated version of VP5 was insoluble; and the use of lower incubation temperatures resulted in only a small increase in solubility. Most of the expressed protein was still present as inclusion bodies. Analysis by western blotting, using anti GST antibodies showed a relatively shorter band than the original truncated VP5.

Hassan et al., (2001); successfully expressed BTV-10 VP5, at high levels and in a soluble form, as an N-terminal GST tagged fusion protein. The use of 'maltose binding protein' (MBP) as a fusion partner was reported to improve membrane protein expression in E. coli. The fusion can be easily cleaved, and this could provide a rational approach for future VP5 expression studies (Hu et al., 2011).

The outer core protein VP7 is involved in cell entry and core particle infectivity in insect cells and adult vector insects. Although VP7 does not raise antibodies that can neutralise intact BTV particles, it can provide partial protection via a cell mediated immune response (Wade-Evans et al., 1996). The association of VP7, when co-expressed with VP2 and VP5, is thought to enhance antigen presentation and the efficacy of VP2 and VP5 vaccines (Wade-Evans et al., 1996; Roy et.al., 1990). Sheep vaccinated with a recombinant capripox virus containing the VP7 gene of BTV-1 were partially protected when challenged with virulent strains of BTV-1 or BTV-3 (Wade-Evans et al., 1996). This protection was achieved in the absence of neutralising antibodies to either the homologous or the heterologous BTV serotypes, indicating the involvement of a protective cell mediated immune response.

BTV-8-VP7 has previously been expressed successfully (Fauziah Mohd-Jaafar personal communication), and is conserved, and is serologically cross-reactive between different BTV serotypes. It was therefore decided to express an alternative VP7, (from BTV-6) in *E. Coli* and for preparation of rMVA vaccines. Blast analysis

suggests that BTV-6 Seg-7 and BTV-8 Seg-7 shares 95 % a.a. identity. Pathak et al., (2008) encountered difficulties in expression of the complete BTV-23 VP7 protein in Prokaryotic systems and therefore expressed a truncated version. BTV-6 Seg-7 encoding VP7 is only 1156 bp long and in the study described here VP7 was successfully expressed in E. coli.

All of the recombinant proteins that were expressed in bacteria in this project were directed to the cytoplasm, which resulted in their expression primarily in the form of insoluble inclusion bodies. This is not a surprise since 30% of proteins from *E. coli* itself cannot be produced in a soluble form when over expressed in E. coli (Graslund et al., 2008). Several attempts were used in this project to reduce the aggregation of the proteins and formation of inclusion bodies. Lower temperature during expression is reported to slow the rate of transcription, translation and refolding of the protein, increasing the quality of the aggregated protein and may even shift the codon usage bias enough to alleviate some expression problems (Vera et al., 2007; Brondyk, W., 2009; Terpe, K 2006; de Groot et al., 2006). However, reducing the temperature was not successful in expressing all the BTV proteins used in this project in a soluble form.
All E. coli expressed proteins in this project were fused to a GST tag (use of solubility-enhancing fusion partner is an attractive alternative to refolding (Nallamsetty, S. and Waugh DS. 2006). Other approaches could have been used to increase solubility of the expressed protein: including fusion to maltose-binding protein (MBP). However, (MBP) often increases the apparent solubility of the recombinant protein, even when the protein is either insoluble by nature, unstable or unfolded and therefore less likely to be active, playing a passive role in the folding of their fusion partners (Graslund et al., 2008; Nallamsetty S and Waugh D. S., 2006) and because proteins that are soluble can be 'dragged' into solution only with a larger tag, but can soon revert to an insoluble form if the fusion partner is removed (Waugh, D. S.2005). A strong lymphoproliferative response to MBP was induced in ponies following inoculation with pure (MBP) (Rachael Chiam, PhD thesis, 2009).

It may be better to use a dual His6-MBP affinity tag. The MBP improves yield and solubility of the protein and the His6- tag facilitates its purification (Waugh, D. S.2005). Removal of the tag can be achieved either by chemical treatment or by use of enzymatic methods. However both methods have their down sides, with some chemical treatments being harsh on the protein and enzymatic treatment requiring a further step to ensure no contaminating protease activity is present in the protein preparation (Arnau et al., 2006),

and removal of the tag could reduce the stability of the expressed protein (Yokoyama, S., 2003) In this project GST tag was not removed.

The formation of inclusion bodies (IBs)can sometimes be an advantage, due to increase in protein stability (Murby et al., 1996), provision of protection from proteases, easily concentrated by centrifugation, minimal contamination with other proteins and the expressed protein is inactive (cannot harm the host) (Murby et al., 1996; Hannig and Makrides 1998; Brondyk, W., 2009). However IBs can also contain significant amounts of properly folded, 'active' protein (Vera et al., 2007; Ventura and Villaverde 2006). To avoid complications by use of soluble and insoluble proteins, all E. coli expressed BTV proteins used in the animal experiments were purified as inclusion bodies. However, in future studies work to improve their solubility could provide a way to increase their immunogenicity as vaccine candidates . The need for further work in this area is indicated by recent studies with BTV-1 proteins expressed a soluble form in bacteria. Although expression of BTV proteins in bacculovirus results in expression of soluble proteins, however this approach was conducted by other colleagues at the Institute for animal health and thus Bacterial expression was used as an alternative method for protein expression which were then used in vaccination trials.

8.3: Vaccination of mice with bacterial expressed proteins

8.2.1 : Pilot study

A vaccination study was conducted to assess the capacity of bacterial expressed BTV proteins (Fragment 1, 2 and 3 of BTV-8-VP2) proteins to BTV-specific antibody responses in Balb/C mice. The bacterially expressed derived proteins were prepared in doses of 60μg or 15μg/ animal formulated with Montanide adjuvant, an amanide-oleate compound that has been shown to produce antibody levels equivalent to Freund's complete adjuvant. All three fragments induced VP2-specific antibodies, as indicated by immune-blotting, irrespective of the dose used (60μg or 15μg/ animal). However, the antisera did not neutralise BTV-8. A possible cause for the failure to generate serum neutralising antibodies was the different conformation of the fragmented VP2 proteins, relative to the native protein. The conformational nature of neutralising epitopes has been demonstrated in earlier studies (DeMaula et al., 2000; Gould and Eaton).

The insolubility of the proteins used and the choice of adjuvant (Montanide) could have had a determinant impact on the immune response. Indeed, Scanlen et al. (2002)

concluded from a study carried out with African horse sickness virus that the induction of virus neutralising antibodies in horses following vaccination with VP2 depends on the protein solubility. This study also concluded that horses were only fully protected against a lethal challenge when VP2 was administered with saponin, and were only partially protected when ISA-50 or AlPO4 were used as adjuvants.

Although serum recovered from mice inoculated with fragmented BTV-8 VP2 failed to neutralise the virus, the VP2 fragments were found to be immunogenic and the antibodies generated in VP2-fragment vaccinated mice proved to be useful in antigen-antibody applications such as immuno-flurocescence and Western blotting.

8.3 : Challenge study

A vaccination and challenge study in IFN-/- knockout mice using a lethal dose of BTV-8, was conducted to investigate the protective capacity of immune responses elicited by bacterial expressed BTV structural proteins (VP2, VP5 and VP7). One of the objectives of this study was to compare the response in mice vaccinated with fragmented VP2+ VP5+ VP7 to those vaccinated with complete VP2+ VP5+ VP7.

All mice vaccinated with the fragmented VP2 showed severe clinical signs, similar to those seen in the control group and were either euthanized or died. In contrast, 50% of mice vaccinated with insoluble VP2 complete, were protected. The absence of protection in the mice vaccinated with the fragmented VP2 protein, correlates with the lack of a virus neutralising antibody response. The lost of conformational epitopes on VP2 due to fragmentation may have played a critical role in the results obtained. Indeed, the correct three dimensional structures of antigens is a critical factor for eliciting antibodies capable of recognising the pathogen upon infection (Stahl and Liljeqvist 1999).

Anti-VP7 immune responses could also play some role in protection, since the 3 mice that were protected in the group vaccinated with complete VP2 +VP5 + VP7, all developed antibodies to VP7 (as detected by immuno-blotting) while the 3 unprotected mice did not.

This study did not measure cellular immunity separately, and it is therefore difficult to assess its significance in the adaptive immune and protective response that was observed. However, VP2 has been identified in other studies, as a target for cytotoxic T lymphocytes in and although protein vaccines are general poor inducers of CTL responses, their potential role in virus clearance should not be disregarded.

GST has a size of 26 KDa and was used as a fusion protein 'tag'. GST is reported to induce anti-GST antibodies; however the level of the anti-GST antibody varies in different mice strains. Antibody responses to GST are low in Balb/C mice, but are intermediate in most other mouse strains, such as CBA/H and C57B1/6 (Davern et al., 1987). As yet there is no published study concerning the anti GST response in IFN-/- knockout mice. However, if GST had an important effect on the immune responses raised to the antigen of interest, this could explain the differences recorded between the groups vaccinated with (Fragmented VP2+ VP5+ VP7) (group 1) or (complete VP2+VP5 +VP7) (group 2) and since each fragmented-protein and VP5 and VP7 used in vaccination were GST-fusion proteins, the mice in group 1 were exposed to a higher concentration of GST in comparison to group2 vaccinated with (complete VP2+VP5 +VP7) thus exposing the mice to a lower concentration of GST. Although tags are believed to increase protein stability, they do have the potential to interfere with the biological activity of the protein and may influence its structure and function (Waugh, D. S., 2005). It would be interesting to extend these protein vaccine studies using less 'bulky'tags or removing them completely from the protein vaccine antigens.

Heterologous DNA prime - protein boost vaccination regimes have been shown to work in some circumstances (Dunham 2002). The success of this strategy is exemplified by studies with West Nile virus. A DNA vaccine encoding the E protein of West Nile virus was protective and the immune responses could be effectively boosted by a recombinant domain DII (Schneeweiss et al., 2011; Lu, S., 2009; Dunham, S. P. 2002). The use of heterologous DNA-prime protein-boost vaccination or a combination including rMVA, may be worth considering in future work with recombinant bluetongue vaccines. However, the order of prime-boost in heterologous vaccination is important. DNA priming may be essential for effective production of antigens *in vivo*, eliciting memory B cells that are specific to conformation domains of an antigen (Shan Lu et al., 2009).

8.4 : DNA vaccines and recombinant MVA vaccines

When administered to an animal, DNA vaccines can direct the *in-vivo* expression of an exogenous / recombinant protein / antigen. Like live attenuated vaccines, DNA vaccines are capable of eliciting both cellular and humoral immune responses, but without the drawbacks of a live attenuated vaccine (Montgomery et al., 1997; Ulmer et

al., 1996). DNA vaccine has been used successfully in a variety of preclinical bacterial, viral, and parasitic animal models (Montgomery et al., 1997).

The main focus of the studies described here was the induction of virus neutralising antibodies and therefore the DNA constructs used encoded the major BTV proteins involved in stimulating virus neutralising antibodies, VP2 VP5) and VP7. However, there is evidence that both VP2 and VP7 can stimulate cellular immunity, which may contribute to protective immunity. In addition, when VP7 is co-expressed with VP2 and VP5, it could potentially improve the conformation of the epitopes presented. This may be more applicable when they are expressed by a single construct (i.e. in the same cell) rather than on separate constructs.

MVA is a well described expression vector that has been used successfully to express viral proteins, with an established safety record (Breathnacha et al., 2003; Calvo-Pinilla et al., 2009a). Recombinant MVA was used in combination with DNA vaccination, or on its own, to determine if it is effective as heterologous DNA prime-MVA boost or MVA-MVA vaccination protocols. This comparison was made since there is some controversy between vaccinologists as to the detrimental effects of pre-existing anti-MVA immunity for the induction of strong immune responses (Moore and Hill 2004). However, Ramiraz et al., (2000), states that repeated inoculation with MVA is possible, due to the low level of MVA replication and circulating anti-vaccinia-virus IgG antibodies in MVA inoculated mice. The, the low neutralising antibodies against the vector may increase the antibody response to the foreign antigen, during subsequent rMVA boost.

Many studies have used DNA-MVA prime boost vaccination (Hanke et.al., 1998, Abaitua et.al., 2006). Both homologous (rMVA-rMVA) and heterologous (DNA-rMVA) prime-boost vaccination regimes, expressing either VP2 alone or a combination of three major BTV proteins VP2, VP5 and VP7, all generated neutralising antibodies by two weeks post boost. The two groups of IFN (-/-) mice vaccinated with VP2 alone, using either DNA-rMVA or rMVA-rMVA, were completely protected against clinical signs of BTV infection and had no detectable viraemia, although a low level of BTV RNA was detected in some individuals by qRT-PCR. This is consistent with the outer capsid protein VP2 being the major neutralization antibody inducer of BTV (Maan et al 2007; DeMaula et.al., 2000; Boone et.al., 2007; Roy et.al.,1990; Huismans et.al., 1987). In our studies, homologous rMVA vaccination using VP2 was as protective as the

heterologous (DNA/rMVA) approach and the protection afforded by VP2 alone was equal or better than the combined expressed proteins.

These results are consistent with previous findings. Studies with recombinant MVA-AHSV-VP2 vaccines, indicated that homologous vaccination induced virus neutralising antibodies in horses and complete protective immunity in a mouse model (Chiam et al., 2009; Castillo-Olivares et al., 2011). However, new findings suggested that prime–boost with different vaccines types, containing the same antigens, can be more immunogenic than a homologous prime–boost strategy (Lu, S., 2009).

A combination of the three major BTV proteins VP2, VP5 and VP7, has been reported to give better protection than VP2 and VP5, or VP2 alone, (Roy et al., 1990; Calvo-Pinilla et.al., 2009a). Indeed co-expression (by recombinant baculovirus) of the four major structural proteins of BTV (VP2, VP3, VP5 and VP7), results in their assembly, generating 'virus like particles' (VLP) that can also raise both neutralising antibodies and a protective response in sheep (Roy P. 1992). However, studies with BTV and other related orbiviruses indicate that complete protection can be achieved by sub-unit vaccines based on VP2 alone (Scanlen et al., 2002, Stone-Marschat et al.,1996). An MVA based vaccine expressing VP2 of African horse sickness virus AHSV Serotype 4 provided complete protection in IFNAR (-/-) mice against homologous AHSV-4 challenge. Vaccination trials using DNA-rMVA expressing VP2, VP5 and VP7 of BTV-4, also protected IFN (-/-) mice from a lethal challenge (Calvo-Pinilla, E., et al., 2009).

There is published evidence that VP5 can influence determinants of BTV neutralisation (DeMaula et.al., 2000 Mertens et al., 1996). However, in the current study, co-expression of BTV-8 VP2, with VP5 and VP7 reduced the overall level of protection in mice. It is important to note that some of the earlier studies involved co-expression in the same cells, from the same virus or constructs. However in the current studies the proteins may be expressed in different cells and may not therefore interact.

The earlier study with BTV-4 also reported that a combination of VP2 and VP5 failed to induce a protective response (Calvo-Pinilla, E., et al., 2009).

Although VP7 does not raise antibodies that can neutralise intact BTV particles, they can neutralise core particles (Hutchinson, I. R., 1999) and can provide partial protection via a cell mediated immune response. The incorporation of VP7 is also thought to enhance the efficacy of VP2 and VP5 vaccines (Wade-Evans et al., 1996; Roy et.al.,

1990). Indeed, VP7 of BTV or AHS alone have both been reported to protect mice from lethal challenge, in the absence of neutralising antibodies (Wade-Evans et al., 1996; Wade-Evans et al., 1997). However, in the current study, vaccination with DNA and / or rMVA expressing VP7 alone did not protect IFN (-/-) mice against BTV-8. This might reflect insufficient antigen generated by either the DNA or the MVA-VP7vaccination dose used ($3x10^5$ pfu). A study by (Santra et al., 2004) suggested that considerably less antigen expression is required to expand an already primed population of memory CTL than to elicit such a population of cells in a naïve host. However, earlier work by (Joost et al., 2009) suggested that doses as low as 10^4 pfu of MVA-HA-VN/04 were sufficient for the induction of protective immunity not only against the homologous strain but also against the antigenically distinct strain of Influenza.

The use of a gene gun (a mechanical method of cell transfection where crossing the plasma membrane is independent of target cell structures) has become increasingly popular for the administration of DNA vaccines (Uchida et al., 2009), This method could potentially reduce the amount of DNA required for prime-vaccination and could be evaluated in future work for the delivery of DNA vaccines (Dunham, S. P 2002). In view of VP7 involvement in protection, by a cell mediated immune response, it was not surprising that no neutralising antibodies were detected in serum of this group of mice (Wade-Evans et al., 1996). It may however be of interest to test the use a higher titre of rMVA-VP7 in future work.

In order to prevent onward transmission of the virus, BTV vaccines should ideally generate sterile immunity, preventing virus replication and viraemia ion the susceptible host. Since clinical BT has never been reported in the absence of viraemia, this would probably also protect vaccinated animals against clinical signs of the disease (Hoffmann et al., 2009). Development of virus-vectored subunit-vaccines, allows the 'live' pathogen to be excluded, the from the production process eliminating disease-security risks. These safe vaccines also prevent or avoid: reversion to virulence (e.g. by reassortment with wild type strains); contamination with toxic compounds used for inactivation; any risk of incomplete inactivation of whole-cell vaccines; and are compatible with DIVA surveillance strategies. Our results show that VP2 expressed in situ using a heterologous or homologous prime boost vaccination (DNA-rMVA or rMVA-rMVA), can generate sterile immunity against BTV-8 in IFN -/- mice, protecting them against a lethal challenge. Since superior efficiency was achieved using VP2

alone, this approach would be of great advantage in reducing the time and resources required for the preparations of more than one recombinant protein.

However, further work will be needed to test and validate the use and efficacy of these BTV-subunit vaccine candidates in ruminants (the natural hosts for BTV infection), including a further investigation of protection against heterologous serotypes and the efficiency of DNA-DNA homologous vaccines and possibly different combinations of DNA/Protein /rMVA. It may also be useful to evaluate the potential of prime / boost vaccinations with VP2 of different serotypes as a way to generate cross-serotype responses and/or protection. This mirrors the cross-protective immune response generated after serial infection with different BTV, eventually generating a response against many serotypes after infection with only three distinct types (Jeggo et al., 1984; 1986). This could be of particular interest in areas where more than one BTV serotype is circulating.

Future work could also investigate the cell-mediated immune response stimulated by different vaccination strategies, generating more valuable information regarding the influence that this arm of immunity has on protection against BTV in the vaccinated animals.

It is however worth noting that throughout the time scale of this project, the animal facilities at IAH were not suitable for animal experiments, this inevitably put a burden on the number of animal groups and the combination used. It also made it impossible to test these vaccination strategies in sheep. Further evaluation using different combinations in mice and sheep would give us valuable information and could set out the new generation of BTV vaccination in UK and Europe.

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10 APPENDIX

10.1 Appendix I: Sequence alignment

10.1.1 a) Sequence alignment file of BTV 9 VP5 Isolate (GRE1999-06)

BTV 9 vp5 Greece showed 99% identicality to that found in the sequence bellow

```
>    emb|AJ586688.1|  Bluetongue virus 9 vp5 gene for outer capsid protein VP5,
genomic
RNA, isolate SER2001/01
Length=1637

 Score = 3009 bits (1629),  Expect = 0.0
 Identities = 1634/1637 (99%), Gaps = 0/1637 (0%)
 Strand=Plus/Plus

Query  1     GTTAAAAAGATCCCCATGATCGCGGAGGATGGGCAAAATCATCAAATCACTGAGCCGTTT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     GTTAAAAAGATCCCCATGATCGCGGAGGATGGGCAAAATCATCAAATCACTGAGCCGTTT  60

Query  61    TGGCAAAAAAGTTGGAGGTGCGTTAACATCTAACACGGCAAAAAAGATCTACAGCACAAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TGGCAAAAAAGTTGGAGGTGCGTTAACATCTAACACGGCAAAAAAGATCTACAGCACAAT  120

Query  121   AGGGAAAGCAGCGGAGAGGTTCGCAGAAAGCGAAATAGGATCAGCAGCCATAGATGGATT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   AGGGAAAGCAGCGGAGAGGTTCGCAGAAAGCGAAATAGGATCAGCAGCCATAGATGGATT  180

Query  181   AGTTCAGGGCAGCGTACATTCCATCATAACTGGCGAGTCTTATGGGGAATCCGTTAAACA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   AGTTCAGGGCAGCGTACATTCCATCATAACTGGCGAGTCTTATGGGGAATCCGTTAAACA  240

Query  241   GGCGGTGTTGCTCAATGTTCTGGGTGCCGGTGATGAAATTCCTGATCCATTGAGTCCAGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GGCGGTGTTGCTCAATGTTCTGGGTGCCGGTGATGAAATTCCTGATCCATTGAGTCCAGG  300

Query  301   TGAGCGTGGGATTCAAATGAAAATTAAGGAGATTGAAGAAGAACAGAGAAATGAGTTAGT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   TGAGCGTGGGATTCAAATGAAAATTAAGGAGATTGAAGAAGAACAGAGAAATGAGTTAGT  360

Query  361   AAGGTTAAAACATGGTAAAGAGATTACAAAAAAGTTTGGGCGGGAATTAGAGGARATATA  420
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  361   AAGGTTAAAACATGGTAAAGAGATTACAAAAAAGTTTGGGCGGGAATTAGAGGAGATATA  420

Query  421   TCAATTCATGAATGGTGAAGTGCGGGAGGAGGCGGAACAGGAGGAACAGTACAAGGTGCT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   TCAATTCATGAATGGTGAAGTGCGGGAGGAGGCGGAACAGGAGGAACAGTACAAGGTGCT  480

Query  481   GTGCAAAGCTGTGGATTCGTACGAGAAGCTATTAGTAGCTGAAAACGAGCAGATGCGAAC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GTGCAAAGCTGTGGATTCGTACGAGAAGCTATTAGTAGCTGAAAACGAGCAGATGCGAAC  540

Query  541   TCTAGCGCGCGCATTACAGAGGGAAACAACAGAGAGAACAGAGACGGAGTCAACGATGGT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   TCTAGCGCGCGCATTACAGAGGGAAACAACAGAGAGAACAGAGACGGAGTCAACGATGGT  600

Query  601   GAAGGAGTACAGACAAAAGATTGATGCCTTGAAGGCGGCTATCGAAGTTGAGAGAGATGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   GAAGGAGTACAGACAAAAGATTGATGCCTTGAAGGCGGCTATCGAAGTTGAGAGAGATGG  660

Query  661   TATGCAGGAAGAAGCTATTCAAGAAATTGCGGGTATGACTGCTGACATATTGGAGGCGGC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   TATGCAGGAAGAAGCTATTCAAGAAATTGCGGGTATGACTGCTGACATATTGGAGGCGGC  720

Query  721   ATCGGAGGAGGTGCCTTTAGTTGGTTCTGGTATGGCTACCGCGATTGCGACTGGGAGAGC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   ATCGGAGGAGGTGCCTTTAGTTGGTTCTGGTATGGCTACCGCGATTGCGACTGGGAGAGC  780
```

```
Query  781   AATCGAAGGAGCATATAAACTCAAGAAAGTAATAAACGCCTTGAGTGGAATCGATCTGTC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   AATCGAAGGAGCATATAAACTCAAGAAAGTAATAAACGCCTTGAGTGGAATCGATCTGTC  840

Query  841   ACATCTGCGAACGCCGAAGATCGAACCTACGATGGTCGCGACGACGTTAGAACATAGGTT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   ACATCTGCGAACGCCGAAGATCGAACCTACGATGGTCGCGACGACGTTAGAACATAGGTT  900

Query  901   TGATGAAATACCGGATAAAGAGTTAGCGGTGAGCATACTAGCAAAGAATAACGCGATAGC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TGATGAAATACCGGATAAAGAGTTAGCGGTGAGCATACTAGCAAAGAATAACGCGATAGC  960

Query  961   TGCGAATACGAGGGAGGTCCAGCATATTAAGGAAGAAATTCTACCAAAATTCAAAAAGAT  1020
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CGCGAATACGAGGGAGGTCCAGCATATTAAGGAAGAAATTCTACCAAAATTCAAAAAGAT  1020

Query  1021  AATGGATGAGGAAAAGGAATTGGAGGGTATAGATGACAAGAAAATCCATCCTAGAGTAAT  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  AATGGATGAGGAAAAGGAATTGGAGGGTATAGATGACAAGAAAATCCATCCTAGAGTAAT  1080

Query  1081  GATGAGATTTAAGGTACCGCGGTCGCAGCAACCGCAGATACATATTTACAGTGCGCCATG  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  GATGAGATTTAAGGTACCGCGGTCGCAGCAACCGCAGATACATATTTACAGTGCGCCATG  1140

Query  1141  GGATTCGGATGACGTGTTCTTTTTTCACTGCGTGTCGCATTTCCACGCCAACGAGTCGTT  1200
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1141  GGATTCGGATGACGTGTTCTTTTTTCACTGCGTGTCGCATTTCCACGCCAACGAGTCATT  1200

Query  1201  CTTTCTAGGGTTCGATTTGGGTATTGATGTCGTTCATTTCGAGGATCTTGCTGCCCACTG  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  CTTTCTAGGGTTCGATTTGGGTATTGATGTCGTTCATTTCGAGGATCTTGCTGCCCACTG  1260

Query  1261  GCACGCTCTGGGCGCCGCACAAGAAGTGAAAGGACGTACCTTGAACGAGGCGTATCGCGA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  GCACGCTCTGGGCGCCGCACAAGAAGTGAAAGGACGTACCTTGAACGAGGCGTATCGCGA  1320

Query  1321  ATTTCTTAATCTGGCGATTAGCAATACGTATACATCTCCAATGCATGCTAGACGTATGAT  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1321  ATTTCTTAATCTGGCGATTAGCAATACGTATACATCTCCAATGCATGCTAGACGTATGAT  1380

Query  1381  AAGGTCGAAAACAGTACACCCCATTTACTTGGGTTCGATGCATTATGATATAACGTATGA  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1381  AAGGTCGAAAACAGTACACCCCATTTACTTGGGTTCGATGCATTATGATATAACGTATGA  1440

Query  1441  AGCATTGAAATCTAACGCACAAAGAATTGTATACGATGACGAATTGCAGATGCACATATT  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1441  AGCATTGAAATCTAACGCACAAAGAATTGTATACGATGACGAATTGCAGATGCACATATT  1500

Query  1501  GAGAGGCCCATTACATTTTCAGCGACGTGCGATATTGGGTGCGCTTAAATTTGGAATTAA  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1501  GAGAGGCCCATTACATTTTCAGCGACGTGCGATATTGGGTGCGCTTAAATTTGGAATTAA  1560

Query  1561  GGTTCTGGGTGACAAAATTGATGTCCCACTCTTCCTACGAAACGCGTGACAGCGGTGATC  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1561  GGTTCTGGGTGACAAAATTGATGTCCCACTCTTCCTACGAAACGCGTGACAGCGGTGATC  1620

Query  1621  CCGGGACTTCCACTTAC  1637
             ||||||||||||||||
Sbjct  1621  CCGGGACTTCCACTTAC  1637
```

10.1.2 b) Sequence alignment file of BTV 16 VP2 Isolate (GRE1999/13)

. BTV 16 VP2 Greece1999/13 showed 99% identicality to that found in the sequence bellow

```
>    emb|AM773702.1|  Bluetongue virus 16 VP2 gene for outer capsid protein, genomic
RNA, strain GRE1999/13
Length=2880
```

Sort alignments for this subject sequence by:

E value Score Percent identity

Query start position Subject start position

```
 Score = 3147 bits (1704),  Expect = 0.0
 Identities = 1723/1736 (99%), Gaps = 0/1736 (0%)
 Strand=Plus/Plus

Query  1     TACGGGCGTGGTATGAGTGGAGCGTAAGACCTGAATATAAACCGCGAGATTTGGAGCGAG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1145  TACGGGCGTGGTATGAGTGGAGCGTAAGACCTGAATATAAACCGCGAGATTTGGAGCGAG  1204

Query  61    AACAAGAAAAATACATCGTTGGGCGCGTTAACCTCTTTGACTTAGAAGGAGAGCCTGCAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1205  AACAAGAAAAATACATCGTTGGGCGCGTTAACCTCTTTGACTTAGAAGGAGAGCCTGCAA  1264

Query  121   CGAAGGTATTTCACTGGGAGTACGAATTGATTAATAAAGTGTATCAGATAACGAATCATA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1265  CGAAGGTATTTCACTGGGAGTACGAATTGATTAATAAAGTGTATCAGATAACGAATCATA  1324

Query  181   CGGGGAATCATTGTGATTTGTATCCTGACRATGTAGAGATCACGGCTAAATTTGATGAGG  240
             |||| |||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1325  CGGGAAATCATTGTGATTTGTATCCTGACGATGTAGAGATCACGGCTAAATTTGATGAGG  1384

Query  241   AGAAATACGGAGAGATGATTCAAACGATAATTAACGAAGGATGGAAGCACGGTGACTTTA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1385  AGAAATACGGAGAGATGATTCAAACGATAATTAACGAAGGATGGAAGCACGGTGACTTTA  1444

Query  301   ARATGTTTAARATTCTGAAGGAGGAGGGTAACCCCTTACTATATGATCTARAGAAGGACA  360
             | |||||||| ||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  1445  AGATGTTTAAGATTCTGAAGGAGGAGGGTAACCCCTTACTATATGATCTAGAGAAGGACA  1504

Query  361   TTAGGTTAGATAGTAGATCACAAGTTATATTTCCACCATATTTCAACAAATGGACGCACG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1505  TTAGGTTAGATAGTAGATCACAAGTTATATTTCCACCATATTTCAACAAATGGACGCACG  1564

Query  421   CTCCAATGTTTAATGCGAAAGTGAAACCATGCGAAGTCGAGTTAGCGCAACGAAAGAATG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1565  CTCCAATGTTTAATGCGAAAGTGAAACCATGCGAAGTCGAGTTAGCGCAACGAAAGAATG  1624

Query  481   AAGACCCTTACGTAAAGCGAACGGTGAAGCCTATACGTGCGGATTGTGTCGATCTGTTAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1625  AAGACCCTTACGTAAAGCGAACGGTGAAGCCTATACGTGCGGATTGTGTCGATCTGTTAA  1684

Query  541   GGTATCACATGTCGCATTACATGGATGTGAGAGTGTCAATGAAGGGGCTTAGTTTAGCGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1685  GGTATCACATGTCGCATTACATGGATGTGAGAGTGTCAATGAAGGGGCTTAGTTTAGCGG  1744

Query  601   TTAAACAGACGCCATCCAGTATTCATCAAGAATTGGCGAAGGATCCATTATATCCATGTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1745  TTAAACAGACGCCATCCAGTATTCATCAAGAATTGGCGAAGGATCCATTATATCCATGTT  1804

Query  661   TTCTGCAGAGGAGAGATGAAAATTTAGATCATAAGTCGGTATGTCCAATCGTCACAAACT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1805  TTCTGCAGAGGAGAGATGAAAATTTAGATCATAAGTCGGTATGTCCAATCGTCACAAACT  1864

Query  721   ATTTCTTATTGGAGAAGTTCTATACTTTAGTTTTAACTATAATGGAAAAGCATTATTGGG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1865  ATTTCTTATTGGAGAAGTTCTATACTTTAGTTTTAACTATAATGGAAAAGCATTATTGGG  1924

Query  781   ATTTAGATGATAGTGACGTTACGTATGAGTTTCCCGCATTGGATGATTCAGCTTACAAGA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1925  ATTTAGATGATAGTGACGTTACGTATGAGTTTCCCGCATTGGATGATTCAGCTTACAAGA  1984

Query  841   CCGAGGGAACGTTGTATGATGTCTCGCAAGTGGTAGTTCATATGATTGATAGATTTTTTG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1985  CCGAGGGAACGTTGTATGATGTCTCGCAAGTGGTAGTTCATATGATTGATAGATTTTTTG  2044

Query  901   AGAAGAGGCGTTTCTTACGCTCAGTCGATGAGTGCAGGTGGATATTGCATTTGGTAAGGT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2045  AGAAGAGGCGTTTCTTACGCTCAGTCGATGAGTGCAGGTGGATATTGCATTTGGTAAGGT  2104

Query  961   CCACCCAAAGTCGGGACAGGCTGAGCGTTTTAAAGCGCTTCTTCCCAACCTTTGGTGAGG  1020
             |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2105  CCACCCGAAGTCGGGACAGGCTGAGCGTTTTAAAGCGCTTCTTCCCAACCTTTGGTGAGG  2164
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Query  1021  GATTGTGTGTAAACAACTTTYGTAAAGTAAAAGATATTATGCTATTAAATTTTCTGCC··  1080
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  2165  GATTGTGTGTAAACAACTTTCGTAAAGTAAAAGATATTATGCTATTAAATTTTCTGCCTT  2224

Query  1081  ···· ··· ·AATCGGTGACAATATAGCGTATGAACATAGACAGTGGGCAGTGCCTTTGC  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2225  TTTTCTTTTTAATCGGTGACAATATAGCGTATGAACATAGACAGTGGGCAGTGCCTTTGC  2284

Query  1141  TYTTYTACGCAGATAAAATAAGGGTTATACCTGCTGAGGTGGGTGCTTATTATAATCGTT  1200
             | || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2285  TCTTCTACGCAGATAAAATAAGGGTTATACCTGCTGAGGTGGGTGCTTATTATAATCGTT  2344

Query  1201  TTGGATTGGTGAGCATCTTAGAGCTGCTAACATTCTTTCCCTCGTATGAAATGCGTGATA  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2345  TTGGATTGGTGAGCATCTTAGAGCTGCTAACATTCTTTCCCTCGTATGAAATGCGTGATA  2404

Query  1261  ATAAGGTTGAGGAGGATGTGCACGCATGCGCGAGCGCGATTCTGGATTTCTATCTGACAA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2405  ATAAGGTTGAGGAGGATGTGCACGCATGCGCGAGCGCGATTCTGGATTTCTATCTGACAA  2464

Query  1321  CAACTATTTCAAATGGAGGTACACAGGCTAGCATAGTTTCAACTAAGGCGTTACTTTATG  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2465  CAACTATTTCAAATGGAGGTACACAGGCTAGCATAGTTTCAACTAAGGCGTTACTTTATG  2524

Query  1381  AGACGTATCTATCCTCGGTGTGTGGCGGTTTTTCTGAAGCGATTCTGTGGTACCTCCCAA  1440
             |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  2525  AGACGTATCTATCCTCGGTGTGTGGCGGTTTTTCTGAGGCGATTCTGTGGTACCTCCCAA  2584

Query  1441  TTACTCATCCAGTTAAGTGTTTAGTCGCGCTTGAGGTTTCGGACTCGCTAGTAGACTCAA  1500
             |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2585  TTACCCATCCAGTTAAGTGTTTAGTCGCGCTTGAGGTTTCGGACTCGCTAGTAGACTCAA  2644

Query  1501  ATGTACGTATCGACAAAATTAAACGAAGATTTCCTTTATCCCACAGGCATTTACGAGGCG  1560
             |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2645  ATGTACGTATCGACTTAATTAAACGAAGATTTCCTTTATCCCACAGGCATTTACGAGGCG  2704

Query  1561  TTGTCCAGATCTCTGTACGGCCAGATCGCACCTTCGGCGTGACGACGTGTGGTATTGTAA  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2705  TTGTCCAGATCTCTGTACGGCCAGATCGCACCTTCGGCGTGACGACGTGTGGTATTGTAA  2764

Query  1621  AGCATAAAATATGTAAAAAGACTTTGTTGAAGCAGCGGTGTGATGTAATTTTAATCCAGA  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2765  AGCATAAAATATGTAAAAAGACTTTGTTGAAGCAGCGGTGTGATGTAATTTTAATCCAGA  2824

Query  1681  TCCCGGGTTACGTTTTCGGTAATGATGAATTACTTACGAAGCTTCTAAATATTTAA  1736
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2825  TCCCGGGTTACGTTTTCGGTAATGATGAATTACTTACGAAGCTTCTAAATATTTAA  2880
```

10.1.3 c) Sequence alignment file of BTV 4 VP5 Isolate (GRE2000/01)

BTV 4 VP5 Greece2000/01 showed 99% identicality to that found in both of the sequences found bellow

```
>    emb|AJ586699.1|  Bluetongue virus 4 vp5 gene for outer capsid protein VP5,
genomic
RNA, isolate RSArrrr/04
Length=1638

                                                    Sort alignments for this
subject sequence by:
                                                      E value  Score  Percent
identity
                                                      Query start position
Subject start position
```

```
 Score = 2146 bits (1162),  Expect = 0.0
 Identities = 1165/1168 (99%), Gaps = 0/1168 (0%)
 Strand=Plus/Plus

Query  1     GTTAAAAAGTGTTCTCCTACTCGCAGAAGATGGGGAAGATAATTAAATCGCTAAGTAGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     GTTAAAAAGTGTTCTCCTACTCGCAGAAGATGGGGAAGATAATTAAATCGCTAAGTAGAT  60

Query  61    TTGGAAAAAAGGTTGGAAACGCATTGTCGTCAAACACAGCGAAGAAAATTTACTCAACCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TTGGAAAAAAGGTTGGAAACGCATTGTCGTCAAACACAGCGAAGAAAATTTACTCAACCA  120

Query  121   TTGGGAAGGCTGCGGAGCGGTTTGCTGAAAGCGAGATCGGTGCGGCGACAATAGATGGCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   TTGGGAAGGCTGCGGAGCGGTTTGCTGAAAGCGAGATCGGTGCGGCGACAATAGATGGCT  180

Query  181   TAGTGCAGGGTAGCGTTCATTCCATAATTACAGGTGAATCTTATGGAGAGTCAGTTAAAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TAGTGCAGGGTAGCGTTCATTCCATAATTACAGGTGAATCTTATGGAGAGTCAGTTAAAC  240

Query  241   AGGCGGTTCTCCTCAACGTGCTAGGTACAGGTGAAGAATTACCAGACCCACTGAGCCCCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   AGGCGGTTCTCCTCAACGTGCTAGGTACAGGTGAAGAATTACCAGACCCACTGAGCCCCG  300

Query  301   GCGAGCGTGGTATGCAGACAAAAATAAAAGAGTTAGAAGACGAGCAGCGAAATGAACTTG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GCGAGCGTGGTATGCAGACAAAAATAAAAGAGTTAGAAGACGAGCAGCGAAATGAACTTG  360

Query  361   TTCGATTAAAGTATAATAAAGAGATAACAAAGGAGTTTGGGAAGGAGCTAGAAGAAGTTT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   TTCGATTAAAGTATAATAAAGAGATAACAAAGGAGTTTGGGAAGGAGCTAGAAGAAGTTT  420

Query  421   ACGACTTCATGAATGGTGARGCGAAGGAGGAGGAAGTAGTTCAGGAACAATACTCAATGC  480
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  421   ACGACTTCATGAATGGTGAGGCGAAGGAGGAGGAAGTAGTTCAGGAACAATACTCAATGC  480

Query  481   TATGCAAAGCAGTAGATTCATATGAGAAAATATTAAAAGCAGAGGACTCGAAAATGGCAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   TATGCAAAGCAGTAGATTCATATGAGAAAATATTAAAAGCAGAGGACTCGAAAATGGCAA  540

Query  541   TATTGGCGCGCGCGTTGCAGCGGGAGGCTTCAGAGAGAAGTCAAGATGAAATCAAAATGG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   TATTGGCGCGCGCGTTGCAGCGGGAGGCTTCAGAGAGAAGTCAAGATGAAATCAAAATGG  600

Query  601   TGAAAGAGTACAGACAGAAGATCGATGCGCTTAAAGATGCGATCGAGATTGAGCGAGATG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TGAAAGAGTACAGACAGAAGATCGATGCGCTTAAAGATGCGATCGAGATTGAGCGAGATG  660

Query  661   GAATGCAGGAGGAGGCGATCCAGGAGATTGCCGGAATGACTGCGGACGTCTTAGAAGCGG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   GAATGCAGGAGGAGGCGATCCAGGAGATTGCCGGAATGACTGCGGACGTCTTAGAAGCGG  720

Query  721   CATCGGAGGAGGTGCCCCTAATTGGCGCGGGTATGGCCACAGYTGTAGCGACCGGTAGAG  780
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  721   CATCGGAGGAGGTGCCCCTAATTGGCGCGGGTATGGCCACAGCTGTAGCGACCGGTAGAG  780

Query  781   CAATAGAAGGCGCATATAAATTGAAGAAAGTCATAAACGCATTGAGCGGGATTGATTTAT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   CAATAGAAGGCGCATATAAATTGAAGAAAGTCATAAACGCATTGAGCGGGATTGATTTAT  840

Query  841   CACATATGAGGAGCCCGAAGATTGAGCCTACTATCATAGCTACAACATTGGAGCATCGAT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CACATATGAGGAGCCCGAAGATTGAGCCTACTATCATAGCTACAACATTGGAGCATCGAT  900

Query  901   TTAAAGAGATACCAGATGAGCAGCTTGCGGTAAGTGTGCTGAATAAAAAGACAGCCGTGA  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TTAAAGAGATACCAGATGAGCAGCTTGCGGTAAGTGTGCTGAATAAAAAGACAGCCGTGA  960

Query  961   CCGATAACTGCAATGAWATTGCGCACATCAAACAGGAAATATTACCAAAGTTTAAGCAGA  1020
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CCGATAACTGCAATGAAATTGCGCACATCAAACAGGAAATATTACCAAAGTTTAAGCAGA  1020

Query  1021  TTATGGATGAAGAGAAGGAGATTGAAGGAATAGAAGATAAAGTGATTCATCCGCGGGTGA  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  TTATGGATGAAGAGAAGGAGATTGAAGGAATAGAAGATAAAGTGATTCATCCGCGGGTGA  1080

Query  1081  TGATGAGGTTCAAAATTCCTAGAACGCAGCAACCGCAAATTCACATTTATGCGGCCCCGT  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  1081  TGATGAGGTTCAAAATTCCTAGAACGCAGCAACCGCAAATTCACATTTATGCGGCCCCGT  1140

Query  1141  GGGATTCTGACGATGTATTTTTCTTTCA  1168
              ||||||||||||||||||||||||||||
Sbjct  1141  GGGATTCTGACGATGTATTTTTCTTTCA  1168


 Score =  785 bits (425),  Expect = 0.0
 Identities = 428/430 (99%), Gaps = 0/430 (0%)
 Strand=Plus/Plus

Query  1179  GGATTCGATCTAGGAATCGATGTCGTTCACTTTGAAGATTTAACCAGCCATTGGCACGCG  1238
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1209  GGATTCGATCTAGGAATCGATGTCGTTCACTTTGAAGATTTAACCAGCCATTGGCACGCG  1268

Query  1239  TTGGGACTAGCACAAGAAGCGAGCGGGCGTACGTTAACGGAGGCGTATCGTGAATTTCTC  1298
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269  TTGGGACTAGCACAAGAAGCGAGCGGGCGTACGTTAACGGAGGCGTATCGTGAATTTCTC  1328

Query  1299  AATTTATCAATCTCAAATACGTATAGTAGCGCTATACATGCGAGGCGTATGATCAGGTCG  1358
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1329  AATTTATCAATCTCAAATACGTATAGTAGCGCTATACATGCGAGGCGTATGATCAGGTCG  1388

Query  1359  CGGGCAGTACACCCTATTTTTCTAGGATCGATGCATTATGATATTACGTATGAAGCTTTA  1418
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1389  CGGGCAGTACACCCTATTTTTCTAGGATCGATGCATTATGATATTACGTATGAAGCTTTA  1448

Query  1419  AAAAATAATGCGCAGAGAATAGTTTATGATGAGGAACTGCAAATGCATATACTAAGGGGA  1478
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1449  AAAAATAATGCGCAGAGAATAGTTTATGATGAGGAACTGCAAATGCATATACTAAGGGGA  1508

Query  1479  CCTTWGCACTTCCAGCGTCGAGCTATTTTAGGGGCGCTGAAATTTGGAGTTAAAATATTA  1538
              |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1509  CCTTTGCACTTCCAGCGTCGAGCTATTTTAGGGGCGCTGAAATTTGGAGTTAAAATATTA  1568

Query  1539  GGCGATAAAATTGATGTCCCCCTCTTCTTACGAAATGCTTGAACGCAACGAGGGAGAAGC  1598
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1569  GGCGATAAAATTGATGTCCCCCTCTTCTTACGAAATGCTTGAACGCAGCGAGGGAGAAGC  1628

Query  1599  TTACACTTAC  1608
              ||||||||||
Sbjct  1629  TTACACTTAC  1638
```

> emb|AJ586681.1| Bluetongue virus 4 vp5 gene for outer capsid protein VP5, genomic
> RNA, isolate GRE2000/01
> Length=1638

Sort alignments for this subject sequence by:

E value Score Percent identity

Query start position Subject start position

```
 Score = 2146 bits (1162),  Expect = 0.0
 Identities = 1165/1168 (99%), Gaps = 0/1168 (0%)
 Strand=Plus/Plus

Query  1     GTTAAAAAGTGTTCTCCTACTCGCAGAAGATGGGGAAGATAATTAAATCGCTAAGTAGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     GTTAAAAAGTGTTCTCCTACTCGCAGAAGATGGGGAAGATAATTAAATCGCTAAGTAGAT  60

Query  61    TTGGAAAAAAGGTTGGAAACGCATTGTCGTCAAACACAGCGAAGAAAATTTACTCAACCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TTGGAAAAAAGGTTGGAAACGCATTGTCGTCAAACACAGCGAAGAAAATTTACTCAACCA  120

Query  121   TTGGGAAGGCTGCGGAGCGGTTTGCTGAAAGCGAGATCGGTGCGGCGACAATAGATGGCT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   TTGGGAAGGCTGCGGAGCGGTTTGCTGAAAGCGAGATCGGTGCGGCGACAATAGATGGCT  180

Query  181   TAGTGCAGGGTAGCGTTCATTCCATAATTACAGGTGAATCTTATGGAGAGTCAGTTAAAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TAGTGCAGGGTAGCGTTCATTCCATAATTACAGGTGAATCTTATGGAGAGTCAGTTAAAC  240

Query  241   AGGCGGTTCTCCTCAACGTGCTAGGTACAGGTGAAGAATTACCAGACCCACTGAGCCCCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  241   AGGCGGTTCTCCTCAACGTGCTAGGTACAGGTGAAGAATTACCAGACCCACTGAGCCCCG  300

Query  301   GCGAGCGTGGTATGCAGACAAAAATAAAAGAGTTAGAAGACGAGCAGCGAAATGAACTTG  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GCGAGCGTGGTATGCAGACAAAAATAAAAGAGTTAGAAGACGAGCAGCGAAATGAACTTG  360

Query  361   TTCGATTAAAGTATAATAAAGAGATAACAAAGGAGTTTGGGAAGGAGCTAGAAGAAGTTT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   TTCGATTAAAGTATAATAAAGAGATAACAAAGGAGTTTGGGAAGGAGCTAGAAGAAGTTT  420

Query  421   ACGACTTCATGAATGGTGARGCGAAGGAGGAGGAAGTAGTTCAGGAACAATACTCAATGC  480
              |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  421   ACGACTTCATGAATGGTGAGGCGAAGGAGGAGGAAGTAGTTCAGGAACAATACTCAATGC  480

Query  481   TATGCAAAGCAGTAGATTCATATGAGAAAATATTAAAAGCAGAGGACTCGAAAATGGCAA  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   TATGCAAAGCAGTAGATTCATATGAGAAAATATTAAAAGCAGAGGACTCGAAAATGGCAA  540

Query  541   TATTGGCGCGCGCGTTGCAGCGGGAGGCTTCAGAGAGAAGTCAAGATGAAATCAAAATGG  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   TATTGGCGCGCGCGTTGCAGCGGGAGGCTTCAGAGAGAAGTCAAGATGAAATCAAAATGG  600

Query  601   TGAAAGAGTACAGACAGAAGATCGATGCGCTTAAAGATGCGATCGAGATTGAGCGAGATG  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TGAAAGAGTACAGACAGAAGATCGATGCGCTTAAAGATGCGATCGAGATTGAGCGAGATG  660

Query  661   GAATGCAGGAGGAGGCGATCCAGGAGATTGCCGGAATGACTGCGGACGTCTTAGAAGCGG  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   GAATGCAGGAGGAGGCGATCCAGGAGATTGCCGGAATGACTGCGGACGTCTTAGAAGCGG  720

Query  721   CATCGGAGGAGGTGCCCCTAATTGGCGCGGGTATGGCCACAGYTGTAGCGACCGGTAGAG  780
              |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  721   CATCGGAGGAGGTGCCCCTAATTGGCGCGGGTATGGCCACAGCTGTAGCGACCGGTAGAG  780

Query  781   CAATAGAAGGCGCATATAAATTGAAGAAAGTCATAAACGCATTGAGCGGGATTGATTTAT  840
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   CAATAGAAGGCGCATATAAATTGAAGAAAGTCATAAACGCATTGAGCGGGATTGATTTAT  840

Query  841   CACATATGAGGAGCCCGAAGATTGAGCCTACTATCATAGCTACAACATTGGAGCATCGAT  900
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CACATATGAGGAGCCCGAAGATTGAGCCTACTATCATAGCTACAACATTGGAGCATCGAT  900

Query  901   TTAAAGAGATACCAGATGAGCAGCTTGCGGTAAGTGTGCTGAATAAAAAGACAGCCGTGA  960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   TTAAAGAGATACCAGATGAGCAGCTTGCGGTAAGTGTGCTGAATAAAAAGACAGCCGTGA  960

Query  961   CCGATAACTGCAATGAWATTGCGCACATCAAACAGGAAATATTACCAAAGTTTAAGCAGA  1020
              |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CCGATAACTGCAATGAAATTGCGCACATCAAACAGGAAATATTACCAAAGTTTAAGCAGA  1020

Query  1021  TTATGGATGAAGAGAAGGAGATTGAAGGAATAGAAGATAAAGTGATTCATCCGCGGGTGA  1080
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  TTATGGATGAAGAGAAGGAGATTGAAGGAATAGAAGATAAAGTGATTCATCCGCGGGTGA  1080

Query  1081  TGATGAGGTTCAAAATTCCTAGAACGCAGCAACCGCAAATTCACATTTATGCGGCCCCGT  1140
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  TGATGAGGTTCAAAATTCCTAGAACGCAGCAACCGCAAATTCACATTTATGCGGCCCCGT  1140

Query  1141  GGGATTCTGACGATGTATTTTTCTTTCA  1168
              ||||||||||||||||||||||||||||
Sbjct  1141  GGGATTCTGACGATGTATTTTTCTTTCA  1168


 Score =  785 bits (425),  Expect = 0.0
 Identities = 428/430 (99%), Gaps = 0/430 (0%)
 Strand=Plus/Plus

Query  1179  GGATTCGATCTAGGAATCGATGTCGTTCACTTTGAAGATTTAACCAGCCATTGGCACGCG  1238
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1209  GGATTCGATCTAGGAATCGATGTCGTTCACTTTGAAGATTTAACCAGCCATTGGCACGCG  1268

Query  1239  TTGGGACTAGCACAAGAAGCGAGCGGGCGTACGTTAACGGAGGCGTATCGTGAATTTCTC  1298
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269  TTGGGACTAGCACAAGAAGCGAGCGGGCGTACGTTAACGGAGGCGTATCGTGAATTTCTC  1328

Query  1299  AATTTATCAATCTCAAATACGTATAGTAGCGCTATACATGCGAGGCGTATGATCAGGTCG  1358
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1329  AATTTATCAATCTCAAATACGTATAGTAGCGCTATACATGCGAGGCGTATGATCAGGTCG  1388
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Query  1359  CGGGCAGTACACCCTATTTTTCTAGGATCGATGCATTATGATATTACGTATGAAGCTTTA  1418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1389  CGGGCAGTACACCCTATTTTTCTAGGATCGATGCATTATGATATTACGTATGAAGCTTTA  1448

Query  1419  AAAAATAATGCGCAGAGAATAGTTTATGATGAGGAACTGCAAATGCATATACTAAGGGGA  1478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1449  AAAAATAATGCGCAGAGAATAGTTTATGATGAGGAACTGCAAATGCATATACTAAGGGGA  1508

Query  1479  CCTTWGCACTTCCAGCGTCGAGCTATTTTAGGGGCGCTGAAATTTGGAGTTAAAATATTA  1538
             |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1509  CCTTTGCACTTCCAGCGTCGAGCTATTTTAGGGGCGCTGAAATTTGGAGTTAAAATATTA  1568

Query  1539  GGCGATAAAATTGATGTCCCCCTCTTCTTACGAAATGCTTGAACGCAACGAGGGAGAAGC  1598
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  1569  GGCGATAAAATTGATGTCCCCCTCTTCTTACGAAATGCTTGAACGCAGCGAGGGAGAAGC  1628

Query  1599  TTACACTTAC  1608
             ||||||||||
Sbjct  1629  TTACACTTAC  1638
```

10.2 Appendix II: BTV-8 segment 2 (Seg-2) complete

GTTAAAATAGCGTCGCGATGGAGGAGCTAGCGATTCCGATTTATACGAATGTATTCCCAGCGGAGCT
TTTAGATGGATATGATTACATCATTGATGTTAGTAGTCGTGTTGAAGAGGAAGGTGATGAGCCCGTT
AAGCGACACGATGTAACAGAGATTCCTAGGAATTCAATGTTCGATATTAAGGATGAACATATACGTG
ATGCGATTATATACAAACCAGTCAACAATGATGGGTACGTACTACCGAGAGTATTAGATATCACGCT
GAAAGCCTTTGACGACCGGAAAAGAGTTGTTTTGAATGATGGTCATAGCGAATTTCATACGAAAACG
AATTGGGTTCAGTGGATGATTGATGACGCAATGGATGTCCAACCTCTTAAGGTAGATATCGCTCACA
CGCGCTCGAGGATCAGCCACGCACTCTTTAACTGTACGGTGCGATTGCATTCAAAAAAGGCTGACAC
CGCATCTTATCACGTTGAACCAGTTGAAATTGAATCGTGGGGATGTAACCACACATGGTTGAGTAGG
ATTCATCATCTAGTGAATGTTGAATTATTTCATTGTTCACAAGAGGCGGCGTATACATTGAAACCCA
CATATAAGATAATATCAAATGCGGAACGTGCATCAACGAGTGATTCCTTTAATGGAACTATGATCGA
ATTAGGTCGTAATCATCAAATTCAGATGGGTGACCAGGGATACCAGAAGTTGAAAGAAGGGTTGGTA
CAAGTTCGAATTGAAGGGAAGACGCCTTTGGTAATACAAGAGGAAATAACCGCTTTGAATAAAATAA
GAGAACAATGGATCGCTCGAAATTTCGATCAAAGGGAAATCAAGGTTTTAGATCTGTGTAGGTTGTT
GTCTACGATAGGTAGGAAGATGTGCAATACTGAAGAGGAACCTAAGAATGAAGCTGATCTTTCAGTG
AAGTTCCAAATGGAGCTTGACGAAATATTTCGACCGGGAAATAACGAGCGTACCAACATCATGGGGG
GCGGAGTACATCGGAAAAACGAGGACAGATTTTACGTACTAATTATGATCGCTGCATCCGATACCAA
TAAAGGTCGTATATGGTGGAGCAATCCGTATCCCTGCCTGCGTGGAGCGTTAATCGCCGCTGAAGTC
CAATTGGGTGATGTTTACAATCTTTTAAGGAATTGGTTCCAATGGAGCGTACGACCTACCTATGTAC
CTTACGATAGGAATAGAGAAAGTGATAAGTATATTTATAGTAGAATCAATTTATTTGATTCAACCCT
GAGACCAGGCGACAAAATAGTACATTGGGAGTATAAGTTGCTTAATGAAGTACGAGAGGTTAGCATC
AACAAAGGTAATGAGTGCGATCTGTTCCCTGAGGATGAAGAATTTACCACCAAGTTTCATGAGGCGC
GGTATACGGAGATGAAGAACCAAATTATTCAAAGCGGGTGGAATCAACGAGATTTTAAAATGCATAA
AATACTAGAGGATGGCGCAAATGTGTTAACGATTGATTTTGAGAAGGACGCGCACATCGGCACGGGT
TCGGCCCTGAGTTTGCCGGATTACTATAACAAATGGATAATTGCTCCGATGTTTAACGCTAAGTTAC
GCATTACCGAGGTTGTGATTGGAACGGCTCACACCGACGATCCAGCTGTAGGGCGCAGCGCGAAGGC
GTTCACACATGATCCTTTTGACCTGCAGCGATATTGTCTAGCAAGATACTATGATGTACGACCTGGT
ATGATGGGCCGCGCTCTCTCTAAGCAACAAAATATGTCGTCGATGACTGATAAACTCTCTAAGCAGG
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GACAGCGCAGTATGTATTCGAGAAAACATGCTTATACGTACTAGAATTATTGAGCAGACATACGATG
CCCTCGGAGGATTCTGAAGTAACGTTTGAACACCCAACAATTGATCCCAGCGTCGATATTGAAACGT
GGAAAATAATTGATGTTTCGCAACTGATCATATTCGTATTTGACTATTTGTTTGAAAATCGGAAGAT
CGTGAGGGATACTACCGAGGCAAGGTGGACGCTTTTCAAAATAAGATCGGAAGTAGGACGAGCCAGG
AATGACGCAATAGAGATGACTTTTCCTCGGTTTGGACGTATGCTAAGAAATGCGTCTCAGGCAAAGA
TAAATCAAGATATCGCGTGCTTGAACTTCCTTCCACTACTTTTCATCATAGGCGATAATATATCTTA
CGCACATAGACAGTGGAGTATACCGGTGCTGCTGTATGCGCACGATATCCGAATTATACCTTTGGAA
GTTGGAGCGTACAATAATCGTTTTGGGCTGACGTCATACCTAGAGTATATGGCTTTCTTTCCATCGT
ACGCTACTAGAGTAGCTAAAATTGACGAGTCGATCAAGGAGTGCGCGATCGCAATGGCTGAGTTTTA
TATGAACACCGATATCCACAGTGGGAGTGTGATGAGTAACGTGATAACGACCAAGCGGCTATTGTAT
GAAACATACTTAGCTTCGCTATGCGGCGGTTATTCGGATGGATTGCTTTGGTACCTGCCAATTACAC
ATCCCTCGAAATGCTTAGTCGCCTTTGAGGTAGCTGATGACGTAGTTCCACTCTCAGTAAGGCGGGA
ACGTATTCTATCTAGGTTTCCTTTAAGCTCGAGACATGTCAAGGGGATTGCGTTGATTTCGGTGGAT
AGGAATCAAAAGGTATCGGTTCAAACAGAGGGTATTGTAACACATCGTCTCTGTAAAAAGAATCTGT
tAAAATACGTGTGTGATGTTATTCTTTTTAAGTTTtCTGGACACGTTTTTGGGAATGATGAGATGTT
AACTAAGCTGCTCAATGTATAGCTCTCGTGACTGAGAGCTCGCGCGCTATCAACTTAC

BTV-8 Seg.2

10.3 Appendix III: BTV-8 segment 2 (Seg-2) fragmentation

GTTAAAATAGCGTCGCGATGGAGGAGCTAGCGATTCCGATTTATACGAATGTATTCCCAGCGGAGCT
TTTAGATGGATATGATTACATCATTGATGTTAGTAGTCGTGTTGAAGAGGAAGGTGATGAGCCCGTT
AAGCGACACGATGTAACAGAGATTCCTAGGAATTCAATGTTCGATATTAAGGATGAACATATACGTG
ATGCGATTATATACAAACCAGTCAACAATGATGGGTACGTACTACCGAGAGTATTAGATATCACGCT
GAAAGCCTTTGACGACCGGAAAAGAGTTGTTTTGAATGATGGTCATAGCGAATTTCATACGAAAACG
AATTGGGTTCAGTGGATGATTGATGACGCAATGGATGTCCAACCTCTTAAGGTAGATATCGCTCACA
CGCGCTCGAGGATCAGCCACGCACTCTTTAACTGTACGGTGCGATTGCATTCAAAAAAGGCTGACAC
CGCATCTTATCACGTTGAACCAGTTGAAATTGAATCGTGGGGATGTAACCACACATGGTTGAGTAGG
ATTCATCATCTAGTGAATGTTGAATTATTTCATTGTTCACAAGAGGCGGCGTATACATTGAAACCCA
CATATAAGATAATATCAAATGCGGAACGTGCATCAACGAGTGATTCCTTTAATGGAACTATGATCGA
ATTAGGTCGTAATCATCAAATTCAGATGGGTGACCAGGGATACCAGAAGTTGAAAGAAGGGTTGGTA
CAAGTTCGAATTGAAGGGAAGACGCCTTTGGTAATACAAGAGGAAATAACCGCTTTGAATAAAATAA
GAGAACAATGGATCGCTCGAAATTTCGATCAAAGGGAAATCAAGGTTTTAGATCTGTGTAGGTTGTT
GTCTACGATAGGTAGGAAGATGTGCAATACTGAAGAGGAACCTAAGAATGAAGCTGATCTTTCAGTG
AAGTTCCAAATGGAGCTTGACGAAATATTTCGACCGGGAAATAACGAGCGTACCAACATCATGGGGG
GCGGAGTACATCGGAAAAACGAGGACAGATTTTACGTACTAATTATGATCGCTGCATCCGATACCAA
TAAAGGTCGTATATGGTGGAGCAATCCGTATCCCTGCCTGCGTGGAGCGTTAATCGCCGCTGAAGTC
CAATTGGGTGATGTTTACAATCTTTTAAGGAATTGGTTCCAATGGAGCGTACGACCTACCTATGTAC
CTTACGATAGGAATAGAGAAAGTGATAAGTATATTTATAGTAGAATCAATTTATTTGATTCAACCCT
GAGACCAGGCGACAAAATAGTACATTGGGAGTATAAGTTGCTTAATGAAGTACGAGAGGTTAGCATC
AACAAAGGTAATGAGTGCGATCTGTTCCCTGAGGATGAAGAATTTACCACCAAGTTTCATGAGGCGC
GGTATACGGAGATGAAGAACCAAATTATTCAAAGCGGGTGGAATCAACGAGATTTTAAAATGCATAA
AATACTAGAGGATGGCGCAAATGTGTTAACGATTGATTTTGAGAAGGACGCGCACATCGGCACGGGT
TCGGCCCTGAGTTTGCCGGATTACTATAACAAATGGATAATTGCTCCGATGTTTAACGCTAAGTTAC
GCATTACCGAGGTTGTGATTGGAACGGCTCACACCGACGATCCAGCTGTAGGGCGCAGCGCGAAGGC
GTTCACACATGATCCTTTTGACCTGCAGCGATATTGTCTAGCAAGATACTATGATGTACGACCTGGT
ATGATGGGCCGCGCTCTCTCTAAGCAACAAAATATGTCGTCGATGACTGATAAACTCTCTAAGCAGG
AGGATTACGCAGGGATTGTTTCAAGGCGTCTGGAATACAAGGAACGAGAGAACAGATGCTTGACGGA
GACAGCGCAGTATGTATTCGAGAAAACATGCTTATACGTACTAGAATTATTGAGCAGACATACGATG
CCCTCGGAGGATTCTGAAGTAACGTTTGAACACCCAACAATTGATCCCAGCGTCGATATTGAAACGT
GGAAAATAATTGATGTTTCGCAACTGATCATATTCGTATTTGACTATTTGTTTGAAAATCGGAAGAT
CGTGAGGGATACTACCGAGGCAAGGTGGACGCTTTTCAAAATAAGATCGGAAGTAGGACGAGCCAGG
AATGACGCAATAGAGATGACTTTTCCTCGGTTTGGACGTATGCTAAGAAATGCGTCTCAGGCAAAGA
TAAATCAAGATATCGCGTGCTTGAACTTCCTTCCACTACTTTTCATCATAGGCGATAATATATCTTA
CGCACATAGACAGTGGAGTATACCGGTGCTGCTGTATGCGCACGATATCCGAATTATACCTTTGGAA
GTTGGAGCGTACAATAATCGTTTTGGGCTGACGTCATACCTAGAGTATATGGCTTTCTTTCCATCGT
ACGCTACTAGAGTAGCTAAAATTGACGAGTCGATCAAGGAGTGCGCGATCGCAATGGCTGAGTTTTA
TATGAACACCGATATCCACAGTGGGAGTGTGATGAGTAACGTGATAACGACCAAGCGGCTATTGTAT
GAAACATACTTAGCTTCGCTATGCGGCGGTTATTCGGATGGATTGCTTTGGTACCTGCCAATTACAC
ATCCCTCGAAATGCTTAGTCGCCTTTGAGGTAGCTGATGACGTAGTTCCACTCTCAGTAAGGCGGGA
ACGTATTCTATCTAGGTTTCCTTTAAGCTCGAGACATGTCAAGGGGATTGCGTTGATTTCGGTGGAT
AGGAATCAAAAGGTATCGGTTCAAACAGAGGGTATTGTAACACATCGTCTCTGTAAAAAGAATCTGT
tAAAATACGTGTGTGATGTTATTCTTTTTAAGTTTtCTGGACACGTTTTTGGGAATGATGAGATGTT
AACTAAGCTGCTCAATGTATAGCTCTCGTGACTGAGAGCTCGCGCGCTATCAACTTAC

BTV-8 Seg.2 showing the different fragments over lapping (fragment one is in yellow, fragment two is underlined, fragment three in red)

10.4 Appendix IV: BTV-8 Seg-6 sequence

```
1                   21                  41                  61                  81
|                   |                   |                   |                   |
GTTAAAAAAGCGATCGCTCTCGCGAAGATGGGGAAAATCATAAAGTCCCTAAGCCGATTCGGAAAGAAAGTTGGAAACGCCTTAACAT
CA
 V  K  K  A  I  A  L  A  K  M  G  K  I  I  K  S  L  S  R  F  G  K  K  V  G  N  A  L  T
S

          101                 121                 141                 161
          |                   |                   |                   |
AACACAGCAAAAAAGATTTATAGCACAATTGGAAAAGCGGCTGAACGATTTGCAGAAAGTGAAATCGGCTCAGCGGCTATTGATGGGT
TA
 N  T  A  K  K  I  Y  S  T  I  G  K  A  A  E  R  F  A  E  S  E  I  G  S  A  A  I  D  G
L

181                 201                 221                 241                 261
|                   |                   |                   |                   |
GTTCAGGGGAGTGTACATTCGTTGATGACGGGAGAGTCTTACGGCGAGTCGGTAAAACAAGCTGTGCTATTAAATGTAATGGGAAGTG
GT
 V  Q  G  S  V  H  S  L  M  T  G  E  S  Y  G  E  S  V  K  Q  A  V  L  L  N  V  M  G  S
G

          281                 301                 321                 341
          |                   |                   |                   |
GAAGAGCTTCCAGATCCACTAAGTCCGGGTGAACGTGGAATGCAGACAAAGATCCGTGAATTGGAGGATGAACAGCGTAATGAGTTGA
TT
 E  E  L  P  D  P  L  S  P  G  E  R  G  M  Q  T  K  I  R  E  L  E  D  E  Q  R  N  E  L
I

361                 381                 401                 421                 441
|                   |                   |                   |                   |
CGGTTGAAGTATAATGATAAGATAAAGCAAAAATTTGGGAAAGAATTAGAAGAGGTATATGAGTTTATGAATGGGGTTGCGAAGCAGG
AG
 R  L  K  Y  N  D  K  I  K  Q  K  F  G  K  E  L  E  E  V  Y  E  F  M  N  G  V  A  K  Q
E

          461                 481                 501                 521
          |                   |                   |                   |
GAAGACGAAGAGAAACATTATGATGTTCTCAAAAAAGCGGTAAACTCGTACGATAAAATCTTAACCGAGGAGGAAAAACAAATGAGGA
TT
 E  D  E  E  K  H  Y  D  V  L  K  K  A  V  N  S  Y  D  K  I  L  T  E  E  E  K  Q  M  R
I

541                 561                 581                 601                 621
|                   |                   |                   |                   |
TTAGCAACAGCGTTACAAAAGGAGGTGAAGGAGCGGACGGGGACGGAGGCCGTCATGGTAAAAGAGTATCGAAATAAGATCGATGCGT
TA
 L  A  T  A  L  Q  K  E  V  K  E  R  T  G  T  E  A  V  M  V  K  E  Y  R  N  K  I  D  A
L

          641                 661                 681                 701
          |                   |                   |                   |
AAAGAGGCCATAGAGGTAGAGCGTGATGGAATGCAGGAAGAGGCGATTCAGGAGATAGCTGGAATGACGGCGGACGTATTGGAGGCGG
CT
 K  E  A  I  E  V  E  R  D  G  M  Q  E  E  A  I  Q  E  I  A  G  M  T  A  D  V  L  E  A
A

721                 741                 761                 781                 801
|                   |                   |                   |                   |
TCGGAGGAAGTGCCACTAATAGGTGCGGGGATGGCGACAGCTGTAGCTACAGGTAGGGCCATCGAAGGAGCTTACAAATTAAAGAAGG
TT
 S  E  E  V  P  L  I  G  A  G  M  A  T  A  V  A  T  G  R  A  I  E  G  A  Y  K  L  K  K
V

          821                 841                 861                 881
          |                   |                   |                   |
ATAAACGCTCTCAGCGGAATCGATTTGACTCACTTAAGAACACCGAAGATTGAGCCTACGATAGTATCGACTGTGTTGGATCATAAGT
TT
 I  N  A  L  S  G  I  D  L  T  H  L  R  T  P  K  I  E  P  T  I  V  S  T  V  L  D  H  K
F

901                 921                 941                 961                 981
|                   |                   |                   |                   |
AAGGATATTCCTGATGAGATGCTCGCGGTAAGCGTTTTATCAAAGAATAGAGCCATCGAAGAGAACCATAAAGAGATTATACATCTTA
AG
 K  D  I  P  D  E  M  L  A  V  S  V  L  S  K  N  R  A  I  E  E  N  H  K  E  I  I  H  L
K
```

```
          1001                1021                1041                1061
          |                   |                   |                   |
AACGAGATATTGCCTAGATTTAAGAAGGCGATGGACGAGGAGAAGGAGATATGTGGAATCGAAGACAAAAAGATACATCCGAAAGTCA
TG
 N  E  I  L  P  R  F  K  K  A  M  D  E  E  K  E  I  C  G  I  E  D  K  K  I  H  P  K  V
M

1081                1101                1121                1141                1161
|                   |                   |                   |                   |
ATGAAATTCAAAATTCCGCGAACTCAACAGCCCCAAATCCATATATATAGTGCACCGTGGGATTCCGACGACGTTTTCTTTTTTCATT
GT
 M  K  F  K  I  P  R  T  Q  Q  P  Q  I  H  I  Y  S  A  P  W  D  S  D  D  V  F  F  F  H
C

          1181                1201                1221                1241
          |                   |                   |                   |
ATATCGCATCACCACGCGAATGAATCATTTTTTATTGGATTTGACTTAGGCATCGATCTGGTTCACTACGAAGATTTGACTGCTCATT
GG
 I  S  H  H  H  A  N  E  S  F  F  I  G  F  D  L  G  I  D  L  V  H  Y  E  D  L  T  A  H
W

1261                1281                1301                1321                1341
|                   |                   |                   |                   |
CACGCTTTGGGTGCGGCGCAGGCGGCGGTTGGGAGGAGTCTGAATGAAGTGTACAAAGAATTTTTGAACCTAGCGATTAATAACACGT
AT
 H  A  L  G  A  A  Q  A  A  V  G  R  S  L  N  E  V  Y  K  E  F  L  N  L  A  I  N  N  T
Y

          1361                1381                1401                1421
          |                   |                   |                   |
AGCTCGCAAATGCACGCTCGGAGGATGATACGATCGAAGACTGTACACCCCATATATCTAGGCTCACTCCATTATGATATTTCGTTTT
CG
 S  S  Q  M  H  A  R  R  M  I  R  S  K  T  V  H  P  I  Y  L  G  S  L  H  Y  D  I  S  F
S

1441                1461                1481                1501                1521
|                   |                   |                   |                   |
ACATTACGGAGCAATGCGCAGAGAATTGTGTATGATGAGGAATTACAGATGCACATATTGAGGGGACCACTACATTTCCAAAGGCGGG
CG
 T  L  R  S  N  A  Q  R  I  V  Y  D  E  E  L  Q  M  H  I  L  R  G  P  L  H  F  Q  R  R
A

          1541                1561                1581                1601
          |                   |                   |                   |
ATACTAGGCGCGATAAAACATGGAGTTAAAATCTTAGGGACGGAGGTTGATATCCCACTCTTCTTAAGAAATGCCTGAGCGCAGCGGA
GC
 I  L  G  A  I  K  H  G  V  K  I  L  G  T  E  V  D  I  P  L  F  L  R  N  A  -  A  Q  R
S

1621
|
CACCGCTTTCCACTTAC
 H  R  F  P  L
```

CGGTTGAAGTATAATGATAAGATAAAGCAAAAATTTGGGAAAGAATTAGAAGAGGTATATGAGTTTATGA
ATGGGGAAAATCATAAAGTCCCTAAGCCGATTCGGAAAGAAAGTTGGAAACGCCTTAACATCAAACACAG
CAAAAAAGATTTATAGCACAATTGGAAAAGCGGCTGAACGATTTGCAGAAAGTGAAATCGGCTCAGCGGC
TATTGATGGGTTAGTTCAGGGGAGTGTACATTCGTTGATGACGGGAGAGTCTTACGGCGAGTCGGTAAAA
CAAGCTGTGCTATTAAATGTAATGGGAAGTGGTGAAGAGCTTCCAGATCCACTAAGTCCGGGTGAACGTG
GAATGCAGACAAAGATCCGTGAATTGGAGGATGAACAGCGTAATGAGTTGATTCGGTTGAAGTATAATGA
TAAGATAAAGCAAAAATTTGGGAAAGAATTAGAAGAGGTATATGAGTTTATGAATGGGGTTGCGAAGCAG
GAGGAAGACGAAGAGAAACATTATGATGTTCTCAAAAAAGCGGTAAACTCGTACGATAAAATCTTAACCG
AGGAGGAAAAACAAATGAGGATTTTAGCAACAGCGTTACAAAAGGAGGTGAAGGAGCGGACGGGGACGGA
GGCCGTCATGGTAAAAGAGTATCGAAATAAGATCGATGCGTTAAAAGAGGCCATAGAGGTAGAGCGTGAT
GGAATGCAGGAAGAGGCGATTCAGGAGATAGCTGGAATGACGGCGGACGTATTGGAGGCGGCTTCGGAGG
AAGTGCCACTAATAGGTGCGGGGATGGCGACAGCTGTAGCTACAGGTAGGGCCATCGAAGGAGCTTACAA
ATTAAAGAAGGTTATAAACGCTCTCAGCGGAATCGATTTGACTCACTTAAGAACACCGAAGATTGAGCCT
ACGATAGTATCGACTGTGTTGGATCATAAGTTTAAGGATATTCCTGATGAGATGCTCGCGGTAAGCGTTT
TATCAAAGAATAGAGCCATCGAAGAGAACCATAAAGAGATTATACATCTTAAGAACGAGATATTGCCTAG
ATTTAAGAAGGCGATGGACGAGGAGAAGGAGATATGTGGAATCGAAGACAAAAAGATACATCCGAAAGTC
ATGATGAAATTCAAAATTCCGCGAACTCAACAGCCCCAAATCCATATATATAGTGCACCGTGGGATTCCG
ACGACGTTTTCTTTTTTCATTGTATATCGCATCACCACGCGAATGAATCATTTTTTATTGGATTTGACTT
AGGCATCGATCTGGTTCACTACGAAGATTTGACTGCTCATTGGCACGCTTTGGGTGCGGCGCAGGCGGCG
GTTGGGAGGAGTCTGAATGAAGTGTACAAAGAATTTTTGAACCTAGCGATTAATAACACGTATAGCTCGC
AAATGCACGCTCGGAGGATGATACGATCGAAGACTGTACACCCCATATATCTAGGCTCACTCCATTATGA
TATTTCGTTTTCGACATTACGGAGCAATGCGCAGAGAATTGTGTATGATGAGGAATTACAGATGCACATA
TTGAGGGGACCACTACATTTCCAAAGGCGGGCGATACTAGGCGCGATAAAACATGGAGTTAAAATCTTAG
GGACGGAGGTTGATATCCCACTCTTCTTAAGAAATGCCTGAGCGCAGCGGAGCCACCGCTTTCCACTTAC

BTV-8 Seg-6 used for cloning in pGEX vector is highlighted in pink

BTV-8 Seg-6 used for cloning in pCI-neo and pSC11 vector is begins at the area highlighted in blue

10.5 Appendix V: BTV-6 NET 2008-04 Seg-7 sequence

```
1                   21                  41                  61                  81
|                   |                   |                   |                   |
GTTAAAAATCTATAGAGATGGACACTATCGCAGCAAGAGCGCTCACTGTAATGCGGGCATGTGCCACTTTACAAGAAGCAAGAATTGT
TT
   -  K  S  I  E  M  D  T  I  A  A  R  A  L  T  V  M  R  A  C  A  T  L  Q  E  A  R  I  V
          101                 121                 141                 161
          |                   |                   |                   |
TGGAGGCAAATGTGATGGAGATATTAGGAATAGCAATCAATAGATATAATGGACTAACTCTACGTGGAGTCACGATGAGACCAACTTC
AT
L  E  A  N  V  M  E  I  L  G  I  A  I  N  R  Y  N  G  L  T  L  R  G  V  T  M  R  P  T  S
181                 201                 221                 241                 261
|                   |                   |                   |                   |
TAGCTCAAAGAAATGAAACGTTTTTTATGTGTTTAGATATGATGCTATCAGCAGCAGGAATCAATGTTGGTCCAATCTCGCCTGACTA
TA
L  A  Q  R  N  E  T  F  F  M  C  L  D  M  M  L  S  A  A  G  I  N  V  G  P  I  S  P  D  Y
          281                 301                 321                 341
          |                   |                   |                   |
```

```
CGCAACACATGGCTACCATAGGTGTGCTGGCGACGCCTGAGATACCGTTCACGACTGAGGCCGCGAATGAGATTGCGCGTGTTACCGG
AG

T  Q  H  M  A  T  I  G  V  L  A  T  P  E  I  P  F  T  T  E  A  A  N  E  I  A  R  V  T  G

361               381               401               421               441

|                 |                 |                 |                 |

AGACTTCAACTTGGGGACCAGCGCGCCAGCCTTACGGTTTTTTCCTTGAAACTGAAGAGGTTTATCAGCCTGGAAGATGGTTTATGCG
AG

E  T  S  T  W  G  P  A  R  Q  P  Y  G  F  F  L  E  T  E  E  V  Y  Q  P  G  R  W  F  M  R

         461               481               501               521

         |                 |                 |                 |

CCGCTCAAGTTGTTACACCAGTGGTTTGTGGACCAGATATGGTTCAGGTTTCGTTGAATGCGGGCGCGAGAGGTGACGTGCAGCAGAT
CT

A  A  Q  V  V  T  P  V  V  C  G  P  D  M  V  Q  V  S  L  N  A  G  A  R  G  D  V  Q  Q  I

541               561               581               601               621

|                 |                 |                 |                 |

TTCAAGGCCGCAATGATCCTATGATGATTTATTTAGTCTGGAGACGGATCGAGAATTTCTCTATGCCGCAGGGTAACTCACAGCGTAC
GC

F  Q  G  R  N  D  P  M  M  I  Y  L  V  W  R  R  I  E  N  F  S  M  P  Q  G  N  S  Q  R  T

         641               661               681               701

         |                 |                 |                 |

TGGCTGGTGTCACTGTGAGCGTAGGTGGTGTGGACATGAGAGCGGGGCGCATAATCGCATGGGATGGACAGGCAGTACTACAAATTCA
CA

L  A  G  V  T  V  S  V  G  G  V  D  M  R  A  G  R  I  I  A  W  D  G  Q  A  V  L  Q  I  H

721               741               761               781               801

|                 |                 |                 |                 |

ATCCGACCCAGCAAAATGCTATGGTGCAAATTCAGGTGGTATTCTACGTTTCTATGGATAAAACGCTTAACCAGTACCCTGCATTGAC
GG

N  P  T  Q  Q  N  A  M  V  Q  I  Q  V  V  F  Y  V  S  M  D  K  T  L  N  Q  Y  P  A  L  T

         821               841               861               881

         |                 |                 |                 |

CAGAAATATTTAATGTGTACAGCTTTAGAGATCACACTTGGCATGGACTAAGGACCGCTATACTAAACAGAACAACCCTTCCAAACAT
GT

A  E  I  F  N  V  Y  S  F  R  D  H  T  W  H  G  L  R  T  A  I  L  N  R  T  T  L  P  N  M

901               921               941               961               981

|                 |                 |                 |                 |

TACCCCCAATCTTTCCGCCGAATGATCGCGATAGTGTACTCACGATCCTACTACTGTCAACGCTCGCAGATGTTTATTCAGTTTTGAG
AC

L  P  P  I  F  P  P  N  D  R  D  S  V  L  T  I  L  L  L  S  T  L  A  D  V  Y  S  V  L  R

         1001              1021              1041              1061
```

```
         |                 |                 |                 |

CTGAGTTCGCAATCCACGGCGTAAATCCAATGCCTGGCCCGCTTACACGTGCGATTGCTCGCGCCGCTTACGCATAGTCCACTTTGCA
CG

P  E  F  A  I  H  G  V  N  P  M  P  G  P  L  T  R  A  I  A  R  A  A  Y  A  -  S  T  L  H

1081              1101              1121              1141

|                 |                 |                 |

GGTGTGGGTTATGCGGGTGGTGTGTCGGTTGCAAGAAATATGTGTCTTGTTTAAACGTCTCTAGATTTACACTTAC

G  C  G  L  C  G  W  C  V  G  C  K  K  Y  V  S  C  L  N  V  S  R  F  T  L
```

```
>6NET 2008-04 orf
GTTAAAAATCTATAGAGATGGACACTATCGCAGCAAGAGCGCTCACTGTAATGCGGGCATGTGCCAC
TTTACAAGAAGCAAGAATTGTTTTGGAGGCAAATGTGATGGAGATATTAGGAATAGCAATCAATAGA
TATAATGGACTAACTCTACGTGGAGTCACGATGAGACCAACTTCATTAGCTCAAAGAAATGAAACGT
TTTTTATGTGTTTAGATATGATGCTATCAGCAGCAGGAATCAATGTTGGTCCAATCTCGCCTGACTA
TACGCAACACATGGCTACCATAGGTGTGCTGGCGACGCCTGAGATACCGTTCACGACTGAGGCCGCG
AATGAGATTGCGCGTGTTACCGGAGAGACTTCAACTTGGGGACCAGCGCGCCAGCCTTACGGTTTTT
TCCTTGAAACTGAAGAGGTTTATCAGCCTGGAAGATGGTTTATGCGAGCCGCTCAAGTTGTTACACC
AGTGGTTTGTGGACCAGATATGGTTCAGGTTTCGTTGAATGCGGGCGCGAGAGGTGACGTGCAGCAG
ATCTTTCAAGGCCGCAATGATCCTATGATGATTTATTTAGTCTGGAGACGGATCGAGAATTTCTCTA
TGCCGCAGGGTAACTCACAGCGTACGCTGGCTGGTGTCACTGTGAGCGTAGGTGGTGTGGACATGAG
AGCGGGGCGCATAATCGCATGGGATGGACAGGCAGTACTACAAATTCACAATCCGACCCAGCAAAAT
GCTATGGTGCAAATTCAGGTGGTATTCTACGTTTCTATGGATAAAACGCTTAACCAGTACCCTGCAT
TGACGGCAGAAATATTTAATGTGTACAGCTTTAGAGATCACACTTGGCATGGACTAAGGACCGCTAT
ACTAAACAGAACAACCCTTCCAAACATGTTACCCCCAATCTTTCCGCCGAATGATCGCGATAGTGTA
CTCACGATCCTACTACTGTCAACGCTCGCAGATGTTTATTCAGTTTTGAGACCTGAGTTCGCAATCC
ACGGCGTAAATCCAATGCCTGGCCCGCTTACACGTGCGATTGCTCGCGCCGCTTACGCATAGTCCAC
TTTGCACGGGTGTGGGTTATGCGGGTGGTGTGTCGGTTGCAAGAAATATGTGTCTTGTTTAAACGTC
TCTAGATTTACACTTAC
```

BTV-6 Seg-7 ORF used for cloning in pGEX and pSC11 vectors is highlighted in red

10.6 Appendix VI: BTV-8 NET 2008-07 Seg-7 sequence

```
1                 21                41                61                81
|                 |                 |                 |                 |
GTTAAAAATCTATAGAGATGGACACTATCGCTGCAAGAGCACTCACTGTGATGCGAGCATGTGCTACGCTTCAAGAAGCAAGAATTGT
GT
   -  K  S  I  E  M  D  T  I  A  A  R  A  L  T  V  M  R  A  C  A  T  L  Q  E  A  R  I  V

         101               121               141               161
          |                 |                 |                 |
TGGAAGCTAACGTGATGGAGATACTAGGGATAGCAATCAACAGATATAATGGATTAACTTTACGAGGGGTGACGATGCGTCCGACTTC
AT
L  E  A  N  V  M  E  I  L  G  I  A  I  N  R  Y  N  G  L  T  L  R  G  V  T  M  R  P  T  S

181               201               221               241               261
|                 |                 |                 |                 |
TGGCGCAGAGAAATGAGATGTTTTTTATGTGTTTAGACATGATGCTGTCCGCTGCTGGAATAAACGTAGGACCGATATCTCCAGATTA
TA
L  A  Q  R  N  E  M  F  F  M  C  L  D  M  M  L  S  A  A  G  I  N  V  G  P  I  S  P  D  Y

         281               301               321               341
          |                 |                 |                 |
CCCAACATATGGCTACAATTGGTGTGCTAGCGACGCCAGAGATACCCTTTACAACGGAAGCGGCGAATGAGATTGCTCGCGTGACAGG
GG
T  Q  H  M  A  T  I  G  V  L  A  T  P  E  I  P  F  T  T  E  A  A  N  E  I  A  R  V  T  G

361               381               401               421               441
|                 |                 |                 |                 |
```

```
AGACTTCAACATGGGGACCAGCGCGCCAGCCCTATGGTTTTTTCCTTGAAACTGAAGAGACTTTCCAACCCGGGAGATGGTTCATGCG
TG
E  T  S  T  W  G  P  A  R  Q  P  Y  G  F  F  L  E  T  E  E  T  F  Q  P  G  R  W  F  M  R

          461                 481                 501                 521
          |                   |                   |                   |
CCGCTCAAGCGGTAACTGCAGTAGTGTGTGGTCCGGATATGATTCAAGTGTCACTGAATGCTGGAGCAAGAGGAGATGTACAGCAGAT
AT
A  A  Q  A  V  T  A  V  V  C  G  P  D  M  I  Q  V  S  L  N  A  G  A  R  G  D  V  Q  Q  I

541                 561                 581                 601                 621
|                   |                   |                   |                   |
TTCAGGGTCGTAACGACCCCATGATGATATATCTAGTTTGGAGAAGAATTGAAAACTTCGCGATGGCGCAGGGTAACTCACAGCAAAC
TC
F  Q  G  R  N  D  P  M  M  I  Y  L  V  W  R  R  I  E  N  F  A  M  A  Q  G  N  S  Q  Q  T

          641                 661                 681                 701
          |                   |                   |                   |
AAGCAGGTGTGACTGTTAGTGTTGGCGGAGTAGATATGCGGGCGGGGCGTATTATAGCGTGGGATGGACAGGCCGCTCTACATGTGCA
CA
Q  A  G  V  T  V  S  V  G  G  V  D  M  R  A  G  R  I  I  A  W  D  G  Q  A  A  L  H  V  H

721                 741                 761                 781                 801
|                   |                   |                   |                   |
ATCCAACACAACAGAATGCAATGGTCCAGATACAAGTTGTGTTTTACATTTCTATGGATAAGACCTTAAATCAATACCCTGCCTTGAC
TG
N  P  T  Q  Q  N  A  M  V  Q  I  Q  V  V  F  Y  I  S  M  D  K  T  L  N  Q  Y  P  A  L  T

          821                 841                 861                 881
          |                   |                   |                   |
CTGAAATCTTTAATGTTTATAGCTTCAGAGATCACACATGGCACGGGTTGAGGACGGCTATACTAAACAGAACTACACTGCCGAATAT
GC
A  E  I  F  N  V  Y  S  F  R  D  H  T  W  H  G  L  R  T  A  I  L  N  R  T  T  L  P  N  M

901                 921                 941                 961                 981
|                   |                   |                   |                   |
TGCCACCTATCTTTCCACCAAACGATCGGGATAGTATTCTGACTCTTTTGCTTTTGTCTACGCTTGCTGACGTTTATACTGTTTTGAG
AC
L  P  P  I  F  P  P  N  D  R  D  S  I  L  T  L  L  L  L  S  T  L  A  D  V  Y  T  V  L  R

          1001                1021                1041                1061
          |                   |                   |                   |
CTGAGTTTGCGATTCATGGCGTAAACCCAATGCCTGGGCCGCTCACACGTGCTATTGCACGCGCCGCCTATGTGTAGTCCACTTTGCA
CG
P  E  F  A  I  H  G  V  N  P  M  P  G  P  L  T  R  A  I  A  R  A  A  Y  V  -  S  T  L  H

1081                1101                1121                1141
|                   |                   |                   |
GGTGTGGGTTACATATGCGGTGTGTCGGTTGTGGGATATATGTAACCCATTCAAACGTCTCTTAGATTACACTTAC
G  C  G  L  H  M  R  C  V  G  C  G  I  Y  V  T  H  S  N  V  S  -  I  T  L
```

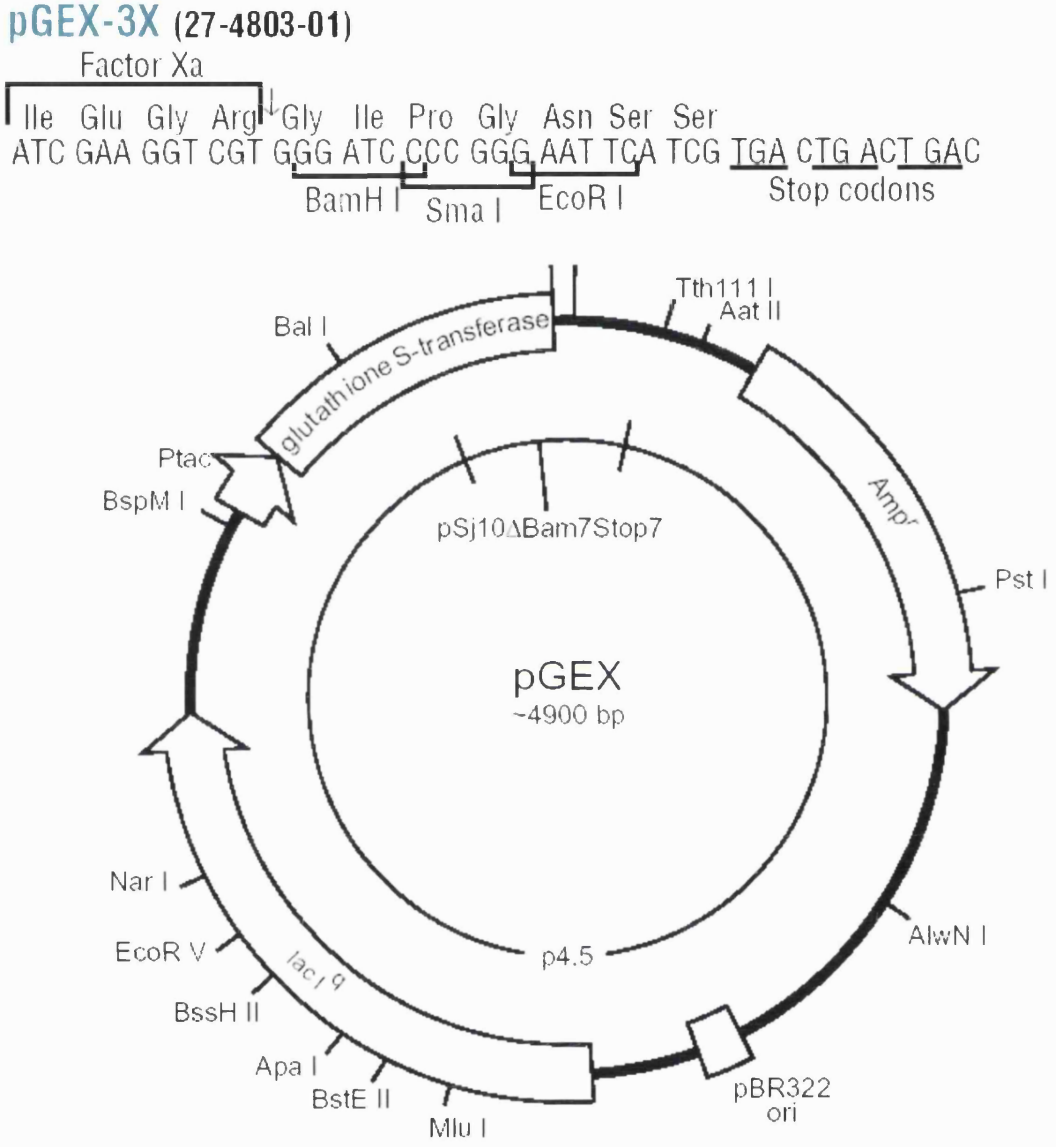
BTV8/VP7 ORF-

GTTAAAAATCTATAGAGATGGACACTATCGCTGCAAGAGCACTCACTGTGATGCGAGCATGTGCTACGCT
TCAAGAAGCAAGAATTGTGTTGGAAGCTAACGTGATGGAGATACTAGGGATAGCAATCAACAGATATAAT
GGATTAACTTTACGAGGGGTGACGATGCGTCCGACTTCATTGGCGCAGAGAAATGAGATGTTTTTTATGT
GTTTAGACATGATGCTGTCCGCTGCTGGAATAAACGTAGGACCGATATCTCCAGATTATACCCAACATAT
GGCTACAATTGGTGTGCTAGCGACGCCAGAGATACCCTTTACAACGGAAGCGGCGAATGAGATTGCTCGC
GTGACAGGGGAGACTTCAACATGGGGACCAGCGCGCCAGCCCTATGGTTTTTTCCTTGAAACTGAAGAGA
CTTTCCAACCCGGGAGATGGTTCATGCGTGCCGCTCAAGCGGTAACTGCAGTAGTGTGTGGTCCGGATAT
GATTCAAGTGTCACTGAATGCTGGAGCAAGAGGAGATGTACAGCAGATATTTCAGGGTCGTAACGACCCC
ATGATGATATATCTAGTTTGGAGAAGAATTGAAAACTTCGCGATGGCGCAGGGTAACTCACAGCAAACTC
AAGCAGGTGTGACTGTTAGTGTTGGCGGAGTAGATATGCGGGCGGGGCGTATTATAGCGTGGGATGGACA
GGCCGCTCTACATGTGCACAATCCAACACAACAGAATGCAATGGTCCAGATACAAGTTGTGTTTTACATT
TCTATGGATAAGACCTTAAATCAATACCCTGCCTTGACTGCTGAAATCTTTAATGTTTATAGCTTCAGAG
ATCACACATGGCACGGGTTGAGGACGGCTATACTAAACAGAACTACACTGCCGAATATGCTGCCACCTAT
CTTTCCACCAAACGATCGGGATAGTATTCTGACTCTTTTGCTTTTGTCTACGCTTGCTGACGTTTATACT
GTTTTGAGACCTGAGTTTGCGATTCATGGCGTAAACCCAATGCCTGGGCCGCTCACACGTGCTATTGCAC
GCGCCGCCTATGTGTAGTCCACTTTGCACGGGTGTGGGTTACATATGCGGTGTGTCGGTTGTGGGATATA
TGTAACCCATTCAAACGTCTCTTAGATTACACTTAC

BTV-8 Seg-7 ORF used for cloning in pCI-neo vectors is highlighted in green

10.7 Appendix VII: Plasmid maps

10.7.1 a) pGEX4T2 vector map (GE Healthcare)



10.7.2 Modified pGEX4T2 sequence

ACGTTATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTAAATC
ACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATAT
TCTGAAATGAGCTG***TTGACAATTAATCATCGGCTCGTATAATGT***GTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG
TATTCATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTGAAGAAA
AATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATC
TTCCTTATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGG
GTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCAT
ATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTC
ATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACC
CAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAAT
CCAG**CAAGTATATAGCATGGCCTTTGC**AGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGGTTC
CGCGTGGATCCCCAG**GAATaCccCTGGAAGTTCTGTTCCAGGGTCCC**GAATTCCCGGGTCGACTCGAGCGGCCGCCATCATCAC
CATCACCATTAGTGA**GCGGCaGC**ATCGTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGA**CGGTGAAAACCTCTGACA**
CATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGT
TGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATAATTCTTGAAGACGAAAGGGCCTCGTGAT
ACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAAC
CCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTG
AAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCA
CCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGG
TAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATC
CCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGA
AAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACT
TCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGA
ACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATT
AACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCG
CTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGG
GCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGC
TGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCA
TTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTG
AGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAA
ACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA
GATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCT
GCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAA
GGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACA
GCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGA
GCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATT
TTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCC
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CCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCT
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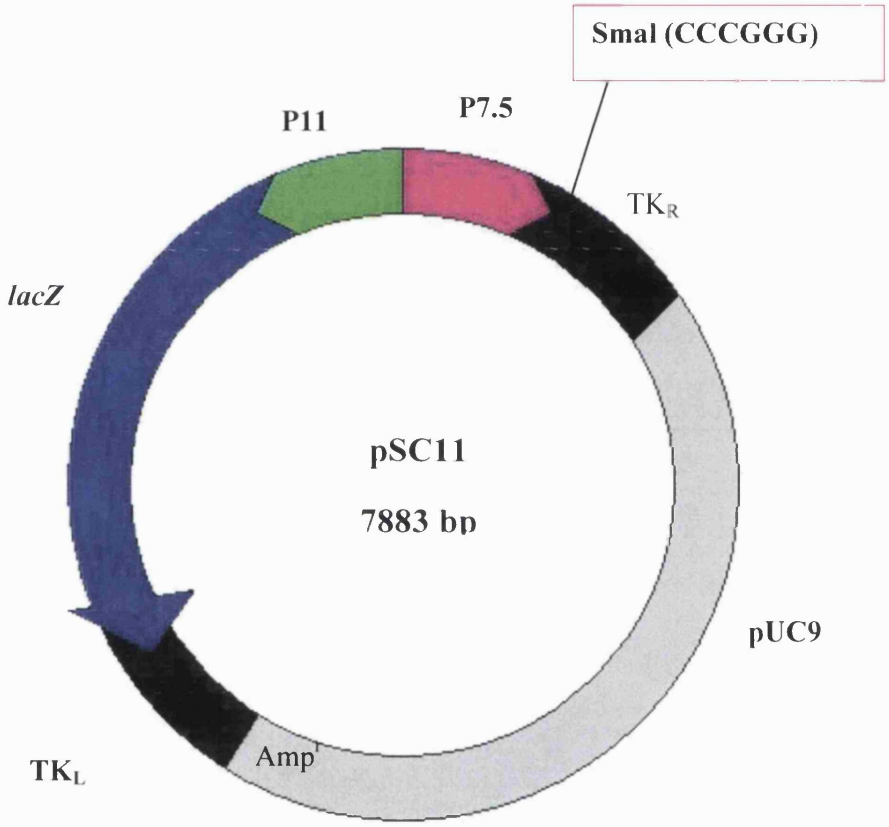
Modified pGEX4T2 sequence

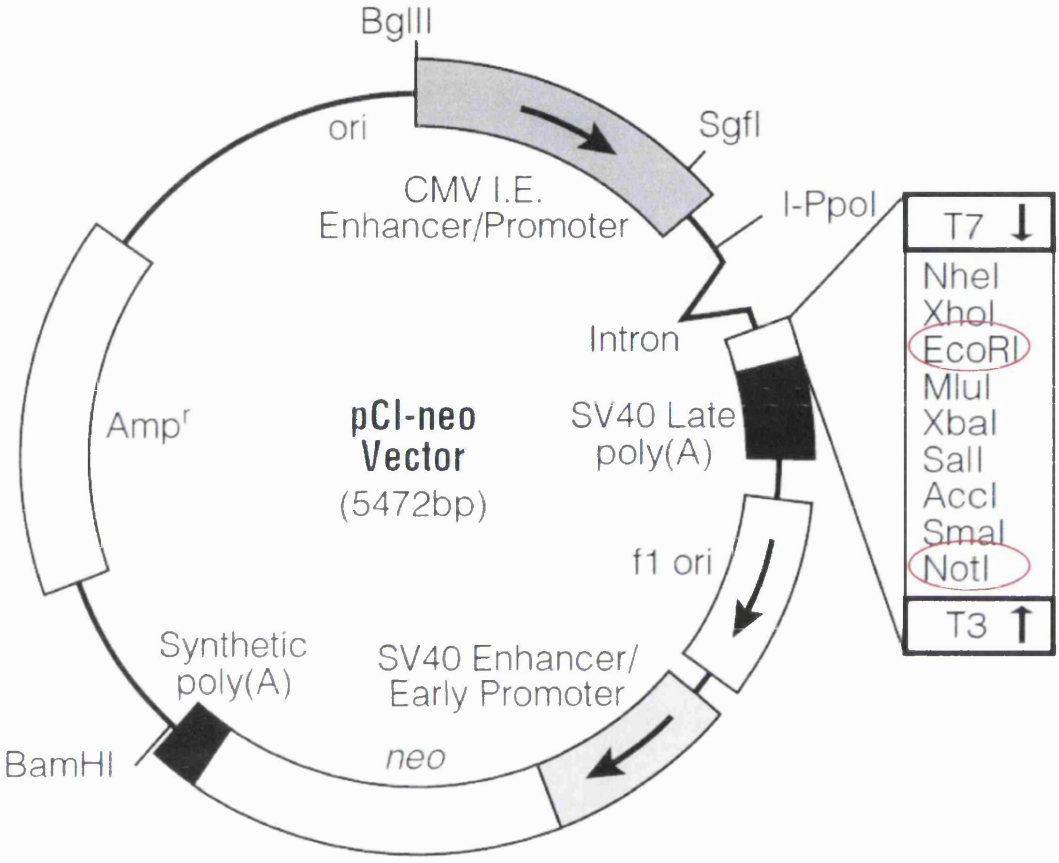
10.7.3 b) pSC11 plasmid map

TK_R and TK_L indicate regions of the thymidine kinase gene required for homologous recombination with the MVA genome (Adapted from Earl and Moss (1992).



pSC11 vector map consisting of the SmaI sequence

10.7.4 c) pCI-neo plasmid map (Promega)



pCI-neo vector map consisting of the XbaI and NotI sequence

10.8 Appendix VIII: General lab protocols and solution preperations

10.8.1 a) LB (Luria-Bertani medium)

deionized H2O	950 ml
tryptone,	10 g
yeast extract	5 g
NaCl	10 g

All components are dissolved in water by placing them in a bottle with a magnet on a magnetic stirrer. The volume of the solution is adjusted to 1 litre with deionised H2O and sterilized by autoclaving

10.8.2 b) Luria Buria (LB) agar plate preperation

deionized H2O	950 ml
tryptone	10 g
yeast extract	5 g
NaCl	10 g
agarose	15 g/litre

All components are desolved in water by placing them in a bottle with a magnet on a magnetic stirrer. The volume of the solution is adjusted to 1 litre with deionised H2O and 15 gm of agarose is added. The bottle is then paced in boiling hot water until the agar is dissolved. The mixture is sterilized by autoclaving, allowed to cool in a water bath set at 50-60°C before adding ampicillin (600 µl/Litre). Plates can then be poured directly from the flask pouring approx. 20-25 ml of medium per 90-mm plate. When the medium has hardened completely, the plates were inverted and stored at 4°C until needed.

10.8.3 c) SOC preparation

For 1 litre 950ml of deionised distal water is mixed with : 20g Tryptone (2% w/v), 5g Yeast extract (0.5%w/v), 2.5 ml 1 M KCl (10 mM), 10 ml 1 M NaCl (10 mM), 10 ml 1 M MgCl2 (10 mM), 10 ml 1 M MgSO4 (10 mM), 10 ml 2 M Glucose (20 mM). For 100 ml of SOC, appropriate amounts of tryptone, yeast extract, NaCl and KCl are added to 97 ml distilled water. The solution is adjusted to pH 7.0 with NaOH and autoclaved. After cooling to room temperature it is filter sterilized and MgCl2, MgSO4 and Glucose are added

10.8.4 d) 2 XYT media

deionized H2O	900 ml

tryptone	16 g
yeast extract	10 g
NaCl	5 g

The volume of the solution is adjusted to 1 litre with deionised H2O and sterilized by autoclaving

10.8.5 e) Qiagen miniprep

Buffer N3: Contains guanidine hydrochloride, acetic acid.

Buffer P2: Contains sodium hydroxide.

Buffer PB: Contains guanidine hydrochloride, isopropanol.

RNase A: Contains ribonuclease.

Principle

The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (1). The unique silica membrane used in QIAprep Miniprep Kits completely replaces glass or silica slurries for plasmid minipreps.

The procedure consists of three basic steps:

- Preparation and clearing of a bacterial lysate
- Adsorption of DNA onto the QIAprep membrane
- Washing and elution of plasmid DNA

10.8.6 f) Preparation of 10% SDS-PAGE gel for protein analysis and electrophoresis

10.8.6.1 1) Separating gel (10% acrylamide)

H2O	6.3 ml
Acrylamide (40%)	4.5 ml
(1.5 M) Tris pH 8.8	4.8 ml
Sucrose 66 % w/w	2.2 ml
SDS 10%	180 µl
APS 10 %	90 µl
TEMED 10%	90 µl

10.8.6.2 2) Concentrating gel (3% acrylamide)

H2O	3.864 ml
Acrylamide (40%)	570 µl
(0.5 M) Tris pH 6.8	1.872 ml
Sucrose 66 % w/w	960 µl
SDS 10%	75 µl
APS 10 %	80 µl
TEMED 10%	120 µl

10.8.7 g) 5x running buffer (1 litre)

Tris	15g
Glycine	72 g
SDS (10%)	50ml
H2O	Complete to 1 litre

10.8.8 h) Denaturing buffer (TSTD)

TSTD composition: 400 µl TS + 100 µl TD; store at -20°C.

10.8.8.1 1) (TS) component

1 M Tris HCl pH 6.8	200mM
EDTA	5 mM
Sucrose	1 M
Bromophenol blue	0.01 %
Methionine	1%

10.8.8.2

10.8.8.3 2) (TD) component

SDS	18%
DTT	0.3 M
ß-mercaptoethanol	1%

10.8.9 i) Western immunoblot:

Buffers and reagents

10.8.9.1 1) Transfer buffer (2 litres)

SDS 10%	10 ml (0.05% final)

Tris	4.8 g (20mM/ l)
Glycine	22 g (150mM/ l)
Isopropanol	400 ml
H2O	Complete to 2 litres

10.8.9.2 2) Washing buffer (TBST)

Tris-HCl pH 7.5	50 mM
NaCl	150 mM
Tween 20	0.1%

10.8.9.3 3) Blocking solution I (Blotto) in PBS

Skim milk	5%
PBS	10ml

10.8.9.4 4) Blocking solution II

Skim milk	5%
NaCl	150 mM
Tris-HCl pH 7.5	50 Mm

10.8.9.5 5) Detection solution

4CN (4-chloro-1-naphthol)	10 ml
H2O2 (30% W/W)	5 µl

10.8.10

10.8.11j) SDS (Sodium Lauryl Sulfate)

To prepare a 10% (w/v) solution 100 g of electrophoresis-grade SDS is, dissolved in 900 ml of H2O. This is heated to 68°C and stired with a magnetic stirrer to assist dissolution. The volume is adjusted to 1 liter with H2O and stored at room temperature.

10.8.12k) IPTG stock solution

Isopropyl-β-D-thiogalactoside (IPTG)	1g
H2O	40 ml

Filter sterilize and store in 2 ml aliquots at -20°C.

10.8.13l) Lysozyme 10 mg/ml

Lysozyme	100 mg
25mM Tris-HCl	10 ml

Store in 5 ml aliquots at -20°C.

10.8.14m) Triton X-100 (20% solution)

Triton X-100	2 ml
PBS solution	8 ml

Rotate overnight to mix. Keep at room temperature

10.8.15n) 4 % Paraformaldehyde

4 % Paraformaldehyde	4 % in PBS

o) 10% (w/v)TCA

Dissolved 20.0g of TCA in deionised water. Make volume up to 200ml

10.8.16p) Crystal violet stain

Dissolve 1 g of crystal violet in 99 g of 20% ethanol. Mix 20 ml of this stock solution with 40 ml of 95% ethanol and 150 ml of water. Store at room temperature.