

The role of Non-Structural Proteins in Bluetongue virus Replication

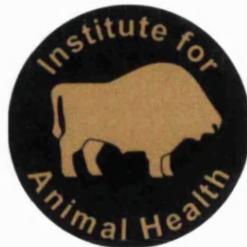
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A thesis submitted in partial fulfilment of the requirements of the
University of London for the degree of
Doctor of Philosophy



These studies were performed at
The Institute for Animal Health
Pirbright Laboratory



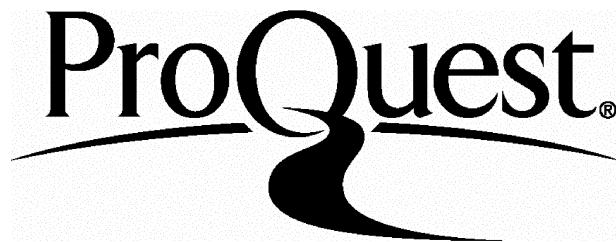
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Declaration

I declare that all of the work submitted herewith has been carried out by myself.
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Andrew Edward Shaw
2009

I. ABSTRACT

Bluetongue virus (BTV) is the type species of the genus *Orbivirus*, family *Reoviridae*. Bluetongue viruses have genomes composed of ten segments of dsRNA, encoding seven structural and four non-structural (NS) proteins. They cause economically important disease (called bluetongue) in ruminants and are transmitted by *Culicoides* biting midges.

The role of BTV NS proteins during replication in mammalian cells was investigated. Genes encoding NS1, NS2 and NS3/3A were cloned and transiently expressed (from transfected plasmids). Localisation of these proteins in uninfected cells matched their distribution in infected cells. However, BTV infection of mammalian cells restricts translation of 'host-like' polyadenylated mRNAs, and restricted expression of NS-proteins from these plasmids. BTV infection also stimulated translation of 'BTV-like' mRNAs, provided they contain both 3' and 5' untranslated regions (UTRs). Specifically, the 3' terminal conserved-hexanucleotide of BTV mRNAs (3'- CAUUCA....) was essential for translation and must be positioned at the terminus, although the remainder of the 3' UTR was also required. However, unlike rotavirus NSP3, BTV-NS2 did not inhibit host cell translation.

BTV infection caused abortive division of mammalian cells, with multiple spindle formation and cell-cycle-arrest in late metaphase. NS2 was seen associated with condensed chromosomes in infected cells displaying aberrant mitosis. NS2 interactions with microtubule-end binding-proteins may prevent association of spindle microtubules with the chromosome kinetochores, blocking mitosis. NS1 was also shown to associate with and disrupt the centrosome.

A real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) assay was developed for detection of BTV and is now a primary diagnostic tool for BTV, used by the community reference laboratory (at IAH Pirbright). The assay was adapted to Qiagen rRT-PCR chemistry and is commercially available.

Double-stranded RNA synthesised *in vitro* was digested with dicer, forming siRNAs. RNA silencing of plasmid expressed mRNAs/protein was achieved, but insufficient time remained to complete these studies in infected cells.

II. ACKNOWLEDGEMENTS

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Appendix III. Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. (2007). Journal of Virological Methods. **145** 115-126.

Appendix IV. Qiagen poster, WAVLD, Madrid, June 2009.

VIII. LIST OF ABBREVIATIONS

A	alanine
AGE	agarose gel electrophoresis
AHSV	<i>African Horse Sickness virus</i>
ATP	adenosine triphosphate
β-ME	β-mercaptoethanol
BHK	baby hamster kidney
BHQ	black hole quencher
BPAEC	bovine pulmonary artery endothelial cells
BSA	bovine serum albumin
BT	Bluetongue
BTV	<i>Bluetongue virus</i>
BVDV	bovine viral diarrhoea virus
C.	<i>Culicoides</i>
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CHO	Chinese hamster ovary
CLP	core-like particle
Cm	centimetre
CMV	cytomegalovirus
CO ₂	carbon dioxide
CPE	cytopathic effect
Ct	threshold cycle
CTL	cytotoxic T lymphocyte
CZ	control zone
CytC	cytochrome C
Da	Dalton
DAPI	4'6'-diamidino-2-phenylindole
DB	dissemination barrier
DIVA	differentiation infected vs. vaccinated animals
DNA	deoxyribonucleic acid
dTNT	deoxynucleotide triphosphate
DPI	days post infection
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EEV	<i>Equine encephalosis virus</i>
EGFP	enhanced green fluorescent protein
EHDV	<i>Epizootic Haemorrhagic Disease virus</i>
EIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbent assay
EM	electron microscope
EU	European Union
FAM	6-carboxyfluorescein
FBS	foetal bovine serum
FMDV	foot and mouth disease virus
FRET	fluorescence resonance energy transfer
g	gram
G	gravity
GMEM	Glasgow's modified eagles medium

GTP	guanosine triphosphate
HRP	horse radish peroxidise
IAH	Institute for Animal Health
IPC	internal positive control
IRES	intrernal ribosomal entry site
ISVP	infectious subviral particles
kDa	kilodalton
l	litre
LB	Luria-Bertani
Lys	lysine
μ	micro
m	milli
M	molar
mA	milliamp
MEVP	membrane enveloped virus particle
MEB	mesenteron escape barrier
MIB	mesenteron infection barrier
MLV	modified live virus
MMLV	molony murine leukaemia virus
MMOH	methyl mercuric hydroxide
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MTOC	microtubule organising centre
MVB	multi-vesicular body
n	nano
nt	nucleotide
NS	non-structural
NTP	nucleotide triphosphate
ORF	open reading frame
P	proline
PABP	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKR	protein kinase R
Poly(A)	polyadenylate
PZ	protection zone
R	arginine
RdRp	RNA dependent RNA polymerase
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
RT	reverse transcription
RT °C	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
RV	rotavirus
S	serine
SAP	shrimp alkaline phosphatase
SCLP	sub-core like particles
SDS	Sodium dodecyl sulphate
Seg	Segment
siRNA	small interfering RNA

SNT	serum neutralisation test
ssRNA	single stranded ribonucleic acid
SZ	surveillance zone
T7p	T7 polymerase
TAMRA	6-carboxytetramethylrhodamine
TCID ₅₀	50% tissue culture infectious dose
TC	transcriptase complex
TOV	Toggenburg orbivirus
USA	United States of America
UTR	untranslated region
V	volts
VIB	viral inclusion body
VLP	virus like particle
VNT	virus neutralisation test
VP	viral protein
v/v	volume/volume
w/v	weight/volume
Y	tyrosine

Chapter 1: Introduction

1.1: Introduction to Bluetongue

Bluetongue virus (BTV) is the type species of the genus *Orbivirus*, within the family *Reoviridae*. Other orbiviruses of veterinary importance include African horsesickness virus (AHSV) and epizootic haemorrhagic disease virus (EHDV). BTV exists as 24 serotypes, with further variations into regional topotypes within individual serotypes. Bluetongue viruses can infect most ruminants and camelids, and have the potential to cause large outbreaks of economically important disease (known as 'bluetongue' (BT)), which particularly affects the 'improved' mutton and fine wool breeds of sheep, certain species of deer, and to a lesser extent in cattle and goats. The clinical signs of BTV infection can vary from inapparent to severe, with estimated case fatality rates in naïve and susceptible sheep populations that have occasionally reached 70 %, as in Cyprus in 1943 (Polydorou, 1978).

BTV is transmitted between its ruminant hosts primarily by adult females of certain 'vector-species' of *Culicoides* biting midge [Diptera: Ceratopogonidae] (Du Toit, 1944). Adult females become infected after feeding on a viraemic mammalian host, and after a period for the virus to disseminate within the insect and infect the salivary glands (the extrinsic incubation period), they can transmit the virus during subsequent blood meals. The virus can also be transmitted vertically in both cattle and sheep, and occasionally via an oral route in cattle (Menzies et al., 2008). BTV also infects some large carnivores after ingestion of infected meat (Alexander et al., 1994; Jauniaux et al., 2008), or injection of contaminated vaccines (Brown et al., 1996).

Ancient 'references' suggest that BT has been a scourge of human society for a long time. Indeed, there are suggestions that the fifth of the 'Ten Plagues of Egypt' as described in the Bible, (Exodus 9:1-7), was due to BTV and AHSV, causing massive losses of ruminants and equids respectively (Marr and Malloy, 1996). Despite such historical references, the first modern record of BTV affecting sheep and cattle, was in South Africa in 1876, where the resulting disease was initially described as 'malarial catarrhal fever' (Hutcheon, 1902). The characteristic (albeit rare) cyanotic appearance of the tongue in infected sheep, led to the later proposal (Spreull, 1905) of the name 'bluetongue'. Since these early descriptions, BTV has been found throughout the world.

The occurrence of BT outbreaks in different regions largely reflects the activity and seasonal / geographic distribution of the adults of competent vector species, and on the rate of infection and virogenesis within the vector insects themselves which is

particularly affected by ambient temperatures (Du Toit, 1944; Mellor, 1990). Recently, climate change and higher temperatures have been linked to dramatic incursions of BT into regions of the world that were never previously affected by BTV, particularly northern Europe (Purse et al., 2008; Purse et al., 2005). Since 1998, there has been a dramatic and progressive increase in the incidence of BT outbreaks in Europe, starting in the Mediterranean region but eventually including the entire region. The repeated introductions of novel BTV strains has caused great economic damage to farming and livestock industries, and has forced a re-evaluation of the potential for arboviral disease outbreaks in the United Kingdom (Wilson and Mellor, 2009).

1.2: Taxonomy

1.2.1: The Family *Reoviridae*

As the type species of the genus *Orbivirus*, *Bluetongue virus* (BTV) represents a basis for much of our understanding of the orbiviruses, and to a lesser extent other reoviruses. BTV was the first orbivirus with a fully sequenced genome. The structure and biochemistry of the BTV virion have also been extensively studied. BTV virus-particles are composed of seven structural proteins, which can be resolved by polyacrylamide gel electrophoresis (in the presence of the detergent SDS, SDS-PAGE) (Mertens et al., 1984). More detailed studies, using cryo-electron microscopy and X-ray crystallography have also resolved the atomic structure of the major components of the BTV core, as well as the organisation of the dsRNA genome segments that it contains, and the outer capsid proteins (Gouet et al., 1999; Grimes et al., 1998; Grimes et al., 1997; Mertens and Diprose, 2004a; Stuart et al., 1998).

1.2.2: The BTV genome

Several virus families have dsRNA genomes, including the *Birnaviridae*, *Cystoviridae*, *Hypoviridae*, *Partitiviridae*, *Totiviridae*, *Chrysoviridae* and *Reoviridae* (Mertens, 2004). Many of these families show generalised similarities, which appear to relate to their replication strategies for a dsRNA genome, and circumvention of dsRNA activated host-cell defences. Members of the family *Reoviridae* contain 10-12 genome segments and represent the largest and most diverse family of the dsRNA viruses (Mertens, 2004).

The reoviruses possess genomes that are composed of 10 to 12 segments of double stranded RNA, with a total molecular weight that ranges from $12 - 20 \times 10^6$ Da, and individual segments that have a molecular weights of $0.2 - 0.3 \times 10^6$ Da, (ICTV,

2005; Joklik, 1983). The reoviruses also share a common replication strategy, based on a transcriptionally active particle (e.g. the BTV core) that carries the entire virus genome into infected cells (Hill et al., 1999; Mertens, 2004), which it then transcribes into mRNA. Despite similarities in their overall structure and replication strategies, there is usually little genetic homology between the members of different genera within the family *Reoviridae* (Calisher and Mertens, 1998; Mertens, 2004).

The genus *Orbivirus* is the largest within the family *Reoviridae* and consists of 20 established species, with 12 further unassigned or ‘tentative’ species (Mertens, 2004). BTV was originally classified as a reovirus, based on electron microscopy which revealed a reovirus-like structure (Studdert et al., 1966), and the subsequent discovery that it possesses a segmented dsRNA genome (Verwoerd and Huismans, 1969). However, further research showed transmission by arthropod vectors, a smaller size, a less clearly defined outer capsid structure, increased sensitivity to variations in pH, temperature and lipid solvents, and the possession of a smooth surfaced core ($T = 13$) that lacks surface projections or ‘turrets’, and several other physiochemical differences from the recognised reoviruses. This led to the recognition of a new genus ‘*Orbivirus*’, with BTV as the type species (Hewat et al., 1992b; Owen and Munz, 1966; Studdert, 1965).

Orbiviruses contain a single copy of each of the dsRNA genome segments within each virus-particle (Mertens et al., 1987a; Mertens et al., 1987b), suggesting certain limitations of the amount of space available within the particle (Gouet et al., 1999). However, recent work using reverse genetics has demonstrated that the BTV particle can artificially package enlarged segments (Matsuo and Roy, 2009), and ‘concatameric’ segments containing duplicated versions of the original sequence have been observed for both Segment (Seg) -9 and Seg-10 of EHDV (Anthony, 2009).

Reovirus genome segments, including those of BTV, can be efficiently separated by polyacrylamide gel electrophoresis (PAGE), providing a high level of resolution, and is even capable of separating segments that have closely similar sizes (Maan, 2004). The migration of dsRNAs during PAGE is not solely dependent on their molecular weight, but is also significantly affected by primary nucleotide sequence. In contrast, agarose gel electrophoresis (AGE) separates the genomic dsRNAs based almost entirely upon their molecular weight and can therefore be used to produce an ‘AGE -electropherotype’ that is characteristic for an individual species or closely related group of reoviruses.

PAGE electropherotyping has been used extensively in BTV research to detect differences between individual strains (Kowalik and Li, 1987; Mertens et al., 1984;

Pedley et al., 1988; Squire et al., 1983a; Squire et al., 1983b). The BTV genome segments have been assigned to the proteins they encode, based upon their consistent migration in 1 % agarose gels and translation of denatured dsRNA *in vitro* (Mertens et al., 1984).

In common with other members of the *Reoviridae*, the majority of the BTV segments are thought to be monocistronic. However two related proteins, VP6 and VP6a, can be produced starting at different initiation codons in Seg-9 (Mertens et al., 1984; Wade-Evans et al., 1992). A further bioinformatic analysis suggests that a second open reading frame may also exist (in the +1 frame) in Seg-9, which would result in a further 9.6 kDa protein (Firth, 2008). However, the importance of this putative protein, if any, is unknown.

Seg-10 of BTV encodes NS3 and NS3A, translated from two in frame initiation codons near to the 5' end of the mRNA, NS3a is identical to NS3, apart from the absence of 13 amino acid residues at its amino terminus (French et al., 1989; Wade-Evans, 1990; Wu et al., 1992). The majority of viral mRNAs contain a favourable 'Kozak consensus sequence' flanking the initiation codon, (Kozak, 1981; Kozak, 1999). Although the downstream NS3A initiation codon is in a 'less favourable' Kozak consensus, translation of both proteins (NS3/NS3a) appears to be approximately equal.

BTV mRNAs contain 5' and 3' untranslated regions (UTRs) either side of the ORF. These UTRs contain inverted repeats of unknown function, although they may be involved in viral mRNA translation (Roy, 1989). A stem loop structure has also been identified within different BTV mRNAs that may be required for RNA binding to non-structural protein 2 (NS2 - (Lymeropoulos et al., 2006). The six terminal nucleotides (hexanucleotides) (5' (GUUAAA) and 3' (CAUUAC) are (usually) identical for all BTV mRNAs, a feature which is also common to other members of the *Reoviridae* (although with different terminal sequences - see http://www.reoviridae.org/dsRNA_virus_proteins/CPV-RNA-Termin.htm ; Mertens and Diprose, 2004; Rao et al., 1983).

The division of orbiviruses (and other reoviruses) into distinct virus species is based primarily on an ability of closely related viruses to exchange genome segments by a process known as reassortment (Mertens, 2000). Reassortment has been widely observed for different BTVs, both in the laboratory and the field (Batten et al., 2008b). However, in the absence of specific data for individual and novel virus isolates, predictions of their reassortment compatibility (species) can be based on other

similarities, identified by structural, biochemical, biological, serological and phylogenetic analyses (ICTV, 2005).

The orbiviruses can be divided into different *serogroups*, which correlates with virus species (e.g. BTV, EHDV and AHSV), and further subdivided into *serotypes* (e.g. the 25 different serotypes of BTV). The different serogroups (species) can be distinguished by serological methods, such as complement fixation, immunodiffusion or enzyme linked immunosorbent assays (ELISA) (Anderson, 1985). Virus-species can also be distinguished and identified by AGE-electropherotype analysis, group specific RT-PCRs, crosshybridisation studies, or by sequencing and phylogenetic analyses of the more conserved genome segments (those encoding the core or non-structural proteins) (Maan, 2004; Shaw et al., 2007).

Most of the virus proteins (with the exception of the outer capsid components – VP2 and VP5 of BTV) are relatively conserved and are serologically cross-reactive between different isolates of the same serogroup/species. However, outer core protein VP7 is immunodominant and consequently represents the major group specific antigen of BTV and related orbiviruses (Gumm and Newman, 1982; Huismans and Erasmus, 1981). Interactions between the different orbivirus serogroups can be complex and some groups/species are more closely related than others, e.g. BTV and EHDV may show low level, or one way serological cross-reactions (Calisher and Mertens, 1998).

In contrast, the identification of individual orbivirus serotypes is based on the specificity of reactions between the virus particle and neutralising antibodies that are generated during infection of the mammalian host (Huismans and Erasmus, 1981). The serotype of the virus isolate, or the specificity of these antibodies can be determined in virus neutralisation tests (VNTs), or serum neutralisation tests (SNTs) respectively (Gould and Eaton, 1990; Mertens, 1994). These antibodies target outer capsid proteins VP2 and VP5, particularly VP2, which contains most of the neutralisation epitopes and varies in a type specific manner (Huismans and Erasmus, 1981; Mertens et al., 1989). Consequently, individual types of BTV, EHDV or AHSV can also be identified by sequencing and phylogenetic analyses, or by RT-PCR assays that target genome segment 2 (Maan et al., 2008; Maan et al., 2007a; Maan, 2009).

Several lines of research, including reassortment analysis, the use of artificially expressed ‘virus like particles’ (VLPs) and modified BTV particles, analysis of neutralising-antibody escape-mutants, and sequence analyses have defined VP2 as the major neutralising antigen that defines BTV serotype (Cowley and Gorman, 1989;

DeMaula et al., 2000; DeMaula et al., 1993; Gould and Pritchard, 1990; Huismans and Erasmus, 1981; Mertens et al., 1989; White and Eaton, 1990). The involvement of VP5 in the determination of serotype is less well defined, although an enhancement of protective antibody responses when it is used as an antigen in combination with VP2 implies that it has a role in defining the serotype (Cowley and Gorman, 1989; Huismans, 1983; Mertens et al., 1989).

Until 2007 there were 24 recognised serotypes of BTV (ICTV, 2005). However in 2007, two flocks of clinically healthy goats in Switzerland were screened for BTV, as part of pre-movement testing, using real-time RT-PCR (targeting Seg-10 (Orru et al., 2006)) and antibody ELISA (detecting antibodies to BTV VP7). At this time, despite the prevalence of BTV-8 across Europe, vaccination was not in use in this area. The real-time RT-PCR curves and serology results were unusually low, leading to further investigation of the flocks. All of the RT-PCR assays used, other than the Orru *et al* assay (2006), failed to produce a positive result, indicating that this was an unusual and potentially novel virus or strain. Experimental infection of goats and sheep with infected blood demonstrated that the virus (designated 'Toggenburg orbivirus' (TOV), after the location in which it had been found) could replicate in goats, albeit without clinical signs (Chaignat et al., 2009). Nucleotide sequencing of the virus genome segments revealed that the virus was closely related to BTV, but different from other known BTV isolates (Hofmann et al., 2008). Interestingly, the level of sequence divergence in Seg-2 (encoding VP2) indicated that although this was a BTV, it represented a distinct 25th serotype (Hofmann et al., 2008; Maan et al., 2007a). Antisera from the infected goats failed to neutralise reference strains of any of the 24 established BTV types, supporting this conclusion.

Initial attempts to isolate TOV and grow it in cell cultures failed, with no detectable cytopathic effect (CPE) in baby hamster kidney (BHK) or Vero cells. However, real-time RT-PCR assays using primers specific for Seg-2 of TOV (N. Maan, personal communication) suggest that the virus may cause a persistent and non-lytic infection of some mammalian cell cultures (S. Maan, personal communication). Without a virus isolate that is adapted to cell culture it is difficult to perform all of the serological and virological assays necessary to definitively include TOV as a new BTV serotype. Furthermore, a virus is required to grow in cell culture if reassortment studies are to be performed in order to demonstrate that TOV is a member of the BTV species.

VP2 and VP5 represent the two most variable BTV proteins. Their presence on the particle surface and interactions with neutralising antibodies is likely to expose them to greater immune pressures, promoting positive selection of antigenic variants and sequence divergence. An alternative (but not mutually exclusive) explanation is that VP2 and VP5, which are the cell attachment and membrane penetration proteins, may also evolve according to the vector and host species / populations present in specific geographical locations.

Most of the BTV segments sequenced so far (can be divided phylogenetically into either an ‘eastern’ or a ‘western’ topotype (Maan et al., 2007a). This even applies to Seg-2 and Seg-6 (encoding outer capsid proteins VP2 and VP5) provided they are considered within an individual virus serotype (Maan et al., 2008; Maan et al., 2007a). Viruses of African or American origin represent the ‘western’ topotype (although with evidence that these represent two western sub-groups), while viruses from India, Malaysia, Australia and Japan represent an ‘eastern’ topotype (Maan et al., 2007a; Mertens, 1994). There is some further evidence of eastern subtypes, particularly involving isolates from China. The recent BT outbreaks in Europe (since 1998) have involved both eastern and western strains (Mellor, 2009).

BTV Seg-7 and Seg-10 can also be subdivided into several different groups (Balasuriya et al., 2008; Maan et al., 2008), which only partially reflect the geographic origins of the virus isolate. VP7 (encoded by Seg-7) and NS3/3A (encoded by Seg-10) are thought to be involved in insect cell entry and exit respectively, and their phylogenetic diversity may therefore reflect regional variations in vector species / populations (K. Nomikou, personal communication).

1.3: BTV proteins and structure

1.3.1: Minor core proteins –VP1, VP4 and VP6

The minor core proteins VP1(Pol), VP4(Cap) and VP6(Hel) together form the replicase complexes within the BTV virus particle. These complexes may explain the non-icosahedrally ordered electron density observed by x-ray crystallography, at the 5 fold axes on the inside the VP3 sub-core layer of BTV core particles (Gouet et al., 1999). The replicase complex is responsible for mRNA transcription and subsequently synthesis of the minus strand of the progeny virus genome. The core contains approximately 12 copies of VP1, each associated with a dimer of VP4 (24 copies per particle) and a hexamer of VP6 (72 copies per particle), to form these replication complexes (Stuart et

al., 1998). Studies with other reoviruses (e.g. cytoplasmic polyhedrosis viruses) indicate that a replication complex is associated with each genome segment (Yazakia and Miura, 1980).

VP1 is encoded by Seg-1 and is the BTV RNA dependent RNA polymerase (Pol) as shown by its expression, purification and RNA polymerase activity (Urakawa et al., 1989). The replicase capability of VP1 was demonstrated by its ability to synthesise a complementary strand when supplied with BTV-like ssRNA representing Segment-10 of the BTV genome (Boyce et al., 2004). In common with other RNA polymerases, three subdomains of the polymerase are arranged in a right hand ‘palm’, ‘fingers’ and ‘thumb’ structure (Wehrfritz et al., 2007). The VP1 protein can be divided into a central GDD motif-containing polymerase domain, flanked by an N-terminal and a C-terminal domain. Remarkably, it has been shown that BTV VP1 polymerase activity can be achieved when the C- N- and polymerase domains are expressed separately and then mixed (Wehrfritz et al., 2007).

The VP1 protein is located within the inner sub-core of BTV, allowing RNA transcription without exposing the viral dsRNA to the host-cell cytoplasm, which could potentially induce dsRNA-activated host defence mechanisms, including PKR and RNA silencing. As is the case for many viral polymerases, Seg-1 (encoding VP1) is the most highly conserved BTV gene (Huang et al., 1995). It also represents the largest of the BTV proteins at 150 kDa (Mertens et al., 1984). Interestingly BTV VP1 shares homology with the polymerase of other RNA viruses, and with bacterial, yeast, insect and DNA-virus RNA-polymerases, e.g. 25 % amino acid identity with vaccinia virus RNA polymerase, as well as subunits of RNA polymerases from *Escherichia coli*, *Saccharomyces* and fruit flies (Roy et al., 1988).

BTV mRNAs contain type-1 methylated cap structures at their 5' termini (identical to those found on eukaryotic mRNAs), which are formed by four sequential enzymatic reactions, all performed by the BTV capping enzyme, VP4(Cap) (Ramadevi et al., 1998a). VP4 is encoded by Seg-4 of the BTV genome and exists as a dimer of the 76 kDa protein. Dimerization is dependent on a ‘leucine-zipper’ domain (Ramadevi et al., 1998b) and, like the other non-turreted members of the *Reoviridae*, is closely associated with VP1 at the particle vertices.

VP4(Cap) has RNA triphosphotase (Ramadevi et al., 1998a) and pyrophosphatase activities (Martinez-Costas et al., 1998). VP4 has two distinct methyltransferase activities (RNA guanine-7-, and RNA 2-O-methyltransferase) as well

as a guanylyltransferase responsible for the 5' capping of the viral messenger RNA (Le Blois et al., 1992; Ramadevi et al., 1998a). Structural studies have revealed that VP4 exists as a rather elongated protein with each capping reaction performed sequentially as the RNA physically passes through the various domains (Sutton et al., 2007).

Because the BTV genome is a double stranded nucleic acid, a helicase is required to unwind the RNA to allow transcription of the genome. Since the polymerase is fully conservative in its action, the newly synthesised mRNA must also be separated from the -ve strand template to allow the original strands of the genome segment to reanneal. VP6 is encoded by the highly conserved Seg-9 and represents the smallest structural protein (35 kDa) with RNA binding domains in the central and C-terminal regions (Hayama and Li, 1994). VP6 possesses both helicase and ATPase activities and may be involved in strand separation during RNA transcription by VP1 (Roy et al., 1990a; Stauber et al., 1997). It has been reported that two forms of VP6 (VP6 and VP6a) exist, although their relative significance has not been investigated (Wade-Evans et al., 1992). There is also some evidence that VP6 forms hexamers in a ring-like structure, a feature characteristic of other helicases (Kar and Roy, 2003).

Reverse genetics has been used to more clearly define the role of VP6 in BTV replication (Matsuo and Roy, 2009). It was shown that VP6 operates early during infection, as rescue attempts failed when Seg-9 RNA (encoding VP6) was eliminated from the first of two transfections 18 hours apart. The helicase activity of VP6 suggests that it may possess an anti- silencing activity by separating the two strands of the siRNAs. This may explain its role and importance in reverse genetics.

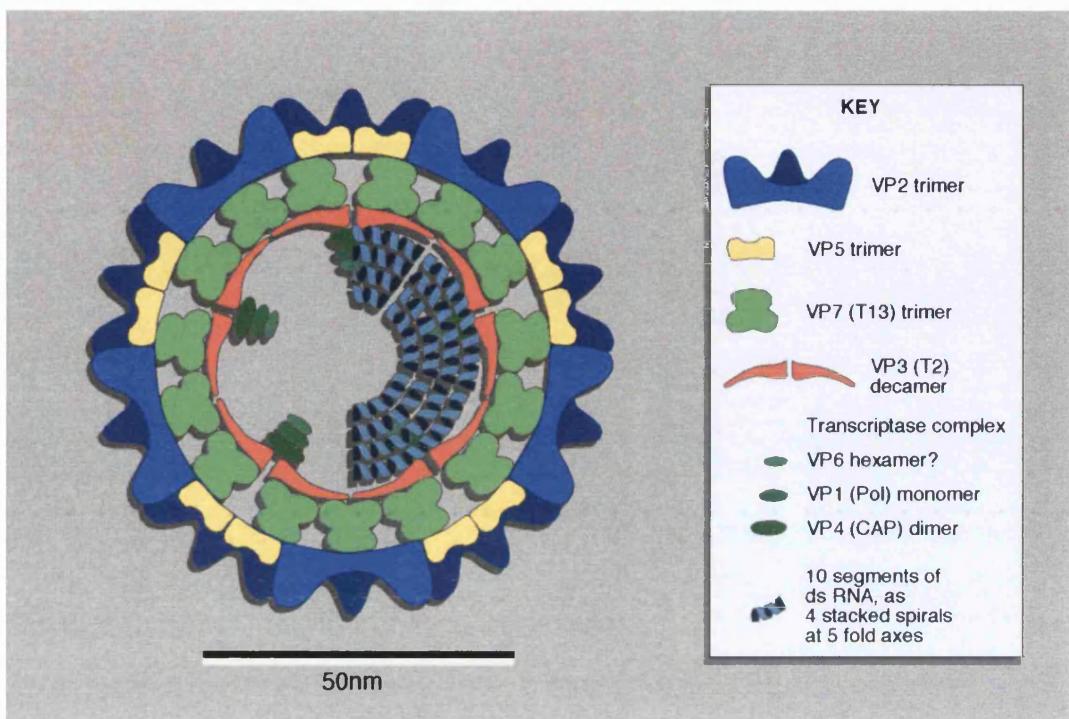


Figure 1. 1: A cartoon of the orbivirus particle.

The dsRNA segments are contained within the core particle associated with the transcriptase complexes (TCs) comprising VP1, VP4 and VP6. The inner sub-core layer of VP3 provides a structure within which the TCs are arranged, and is stabilised by an outer-core layer of VP7. The core is subsequently encased in the outer coat proteins VP2 and VP5. Published with permission, Mertens, (2002).

1.3.2: core structure proteins – VP3 and VP7

VP3 is a 110 kDa protein encoded by Seg-3, and represents the inner-most shell of the core particle. 120 copies of VP3 can self-assemble into a the innermost capsid shell of BTV, with $T = 2$ symmetry, which is surrounded by the core-surface layer composed of 780 molecules of VP7 arranged as 260 trimers, with $T=13/$ symmetry (Figure 1.1) (Grimes et al., 1998). Initially, monomers of VP3 assemble into flower-like, dish-shaped decamers. Twelve decamers (one centred on each of the five-fold icosahedral axes) then assemble, interacting via their ‘zig-zag’ outer edges, to form a smooth, thin sub-core particle, which acts as a scaffold for the attachment of further core components (Grimes et al., 1998).

During replication, VP3 localises primarily to the viral inclusion bodies (VIB - large granular ‘matrices’, which represent the site of viral mRNA synthesis, replication and progeny virus assembly within the cytoplasm of infected cells). It interacts via its carboxy-terminus with other viral proteins, particularly VP7 (Kar et al., 2005). The arrangement of VP3 in the BTV sub-core closely reflects equivalent structure in other reoviruses, leading to a suggestion that the correct assembly of the VP3 layer is of critical

importance for these viruses (Grimes et al., 1998; Reinisch et al., 2000). Despite the obvious structural conservation between different viruses, modified versions of VP3(T2) can still form core-like particles (CLP) when expressed with VP7(T13) (Tanaka et al., 1995; Tanaka and Roy, 1994).

X-ray crystallography studies indicate that chemically identical VP3 molecules adopt one of two conformations within the BTV sub-core layer, which are identified as 'A' and 'B' by Grimes et al (1998). Transition between the A and B forms involves a number of simple conformational changes, with particular regard to the flexibility of the N-terminal region (Gouet et al., 1999; Grimes et al., 1998). The N-terminal 10 residues and the 'dimerization domain' of VP3 are critical for assembly of core-like particles (Kar et al., 2004). Deletion of the dimerization domain of VP3 also abrogated its interaction with RNA, on the inside of the core (Kar et al., 2004).

VP7 is a hydrophobic 38 kDa protein encoded by BTV Seg-7 and represents the outermost core protein. The core surface comprises 260 VP7 trimers (780 copies per particle) arranged with $T=13/1$ icosahedral symmetry (Basak et al., 1992). In replicating cells VP7 is observed in the viral inclusion bodies (Brookes et al., 1993). The precise role of VP7 in virus replication remains somewhat obscure as it has recently been found that VP7 is not required in order to form a particle with replicase activity (Matsuo and Roy, 2009). However, the VP7 shell enhances the stability, rigidity and strength of the core particle (Grimes et al., 1995; Grimes et al., 1998). The C-terminus of VP7 is required for the assembly of the core, and replacement of Lys-255 with serine abrogated CLP formation (Le Blois and Roy, 1993).

VP7 trimers have an upper and a lower domain. The upper domain (amino acids 121-249) forms the outermost surface of the core particle and interacts with outer capsid proteins VP2 and VP5 (Grimes et al 1998). The upper domain of VP7 also contains an arginine-glycine-aspartic acid (RGD, amino acids 168-170) motif. RGD motifs are known to bind integrin receptors, and since core particles are themselves infectious for *Culicoides* cells (Mertens et al 1996), may be involved in cell-attachment (Basak et al., 1996; Tan et al., 2001; Xu et al., 1997). The RGD motif is itself conserved among all BTV strains examined, with the exception of BTV-15 from Australia (Wang et al., 1994), although this strain has a different RGD motif also in the 'head-region' of VP7. The lower domain of VP7 interacts with the VP3 sub-core layer in 13 distinct orientations and contains a number of residues that are critical for core formation, but not for VP7 trimerization (Limn et al., 2000). CLPs were formed when VP3 and VP7 were expressed

together from recombinant baculoviruses, demonstrating that these proteins alone are sufficient to form the capsid of the core (French and Roy, 1990).

An interesting property of VP7 is its ability to bind dsRNA in a manner that is independent of nucleotide sequence (Diprose et al., 2002). This has the potential to sequester any dsRNA released during BTV replication and might provide a way of avoiding activation of dsRNA-dependent host-cell defence-mechanisms, e.g. RNA silencing responses.

The nucleotide sequence of Seg-7 (encoding VP7) appears to be more variable than the genome segments encoding other core proteins, and can be divided into a number of ‘groups’ that only partially reflect the geographic origins (topotype) of the virus isolate. However, VP7 itself is more highly conserved reflecting a large number of conservative changes, particularly in the 3rd base position in the Seg-7 open reading frame (ORF). This suggests that variants of VP7 have been maintained in different strains of the virus over very long periods of time, suggesting that their differences may have some functional significance. The role of VP7 in the infection of insect vector cells itself suggests that these variations may reflect the use of different insect populations as vectors. VP7 is immunodominant and represents the major group specific antigen of BTV that is used in serological assays (Eaton et al., 1991; Gumm and Newman, 1982; Wang et al., 1996).

1.3.3: Outer capsid proteins – VP2 and VP5

VP2 and VP5 (encoded by Seg-2 and Seg-6 respectively) assemble on the outer VP7 layer of the core, to form the outer capsid layer of the virus particle, with VP2 somewhat obscuring VP5 (Hyatt et al., 1993). The VP2 monomer is 111 kDa with 180 copies per particle in the form of 60 trimers (Hewat et al., 1992b).

VP2 is the primary determinant of BTV serotype and is the most variable of the viral proteins (Mertens et al., 1989), with VP5 the second most variable (Roy, 1989; Singh, 2005). Seg-2 has therefore been of great interest for phylogenetic and molecular epidemiological studies (Balumahendiran et al., 2009; Dahiya et al., 2005; Maan et al., 2007a; Potgieter et al., 2005; Pritchard and Gould, 1995; Yamakawa et al., 1994). VP2 contains several different neutralisation epitopes, binding sites for antibodies that can block the initiation of infection of BTV or AHSV (Burrage et al., 1993; Cowley and Gorman, 1989; Huismans and Erasmus, 1981; Hwang and Li, 1993).

In addition to its role in serotype determination, VP2 is thought to be responsible for the initial binding of the virus particle to the cell membrane (Hassan and Roy, 1999),

as well as a postulated interaction with vimentin during virus egress (Bhattacharya et al., 2007). The VP2 protein is responsible for haemagglutination of ovine erythrocytes (Cowley and Gorman, 1987; Cowley and Gorman, 1990). Haemagglutination is thought to arise from BTV binding to serine-linked oligosaccharides in erythrocyte glycophorins (Eaton and Crameri, 1989) and can be blocked by cleavage of VP2 in ‘infectious subviral particles’ (ISVP) (Mertens *et al* 1987a).

Each BTV particle contains 360 copies of VP5 (molecular weight of 59kDa) arranged as 120 trimers, somewhat underlying and interspersed between the VP2 trimers (Stuart et al., 1998). VP5 also contains sequences that influence the specificity virus interactions with neutralising antibodies, possibly via an association with VP2 and clearly has a role in the overall neutralisation characteristics (serotype) of the BTV particle (DeMaula et al., 2000; Mertens et al., 1989). VP5 is glycosylated, although the glycosylation branching appears to be short (Yang and Li, 1993).

The N-terminal domain of VP5 contains coiled-coil structures, similar to the fusion proteins of influenza virus A haemagglutinin, the paramyxovirus F proteins and gp41 of human immunodeficiency virus, and is (at least in part) responsible for cellular cytotoxicity (Hassan et al., 2001). Expressed VP5 can cause cell fusion and syncitium formation (at both pH 5.5 and pH 7.5) and it may therefore be involved in penetration of the cell membrane (Hassan et al., 2001). These properties, along with the presence of stretches of hydrophobic amino acids, have led to the suggestion that VP5 acts in a manner reminiscent of other fusion proteins.

Recent data relating to floatation experiments have indicated that the interaction between VP5 and a SNARE domain/lipid raft are at least partially responsible for its fusion properties (Bhattacharya and Roy, 2008). VP7 and VP5 are capable of causing apoptosis in infected cells. However, the apoptotic response is only induced in mammalian cells and occurs only when the proteins are applied extracellularly and together (Mortola et al., 2004). Characteristic apoptotic responses, (cellular DNA fragmentation, morphological changes, caspase-3 activation and activation of the transcription factor NF- κ B) were not observed when the proteins were expressed intracellularly or applied individually to the cell surface (Mortola et al., 2004). These data may relate to a study of AHSV reassortants where it was demonstrated that the outer capsid proteins were an important determinant of virulence in a mouse model (O’Hara et al., 1998).

1.3.4: Forms of the virus particle

The multi-layer particle is characteristic of many members of the family *Reoviridae* (although cypoviruses have a single shelled capsid structure). Certain modification of each capsid layer can result in several different forms of the orbivirus particle (Mertens et al., 1987a). The BTV particles can exist in at least five forms. These include membrane enveloped virus particles (MEVP), which are released by budding from infected mammalian or insect cells. The membrane layer of these particles is thought to be relatively unstable and is lost during purification (Mertens et al., 1987a). They have not therefore been extensively studied or characterised. However, a large proportion of the virus that is generated in infected cell cultures is highly membrane associated (Hyatt et al., 1989).

Complete and intact BTV virus particles are usually regarded as non-enveloped and non-lipid-containing, approximately 80 nM in diameter, and are competent to enter (infectious for) both mammalian and insect cells (Mertens et al., 1987a). The complete removal of the outer capsid proteins by a low pH environment (e.g. an endosome), or by metal ion treatments (Mg^{2+} or $CsCl$ gradient centrifugation) results in release of the core particle and activation of the virus-core-associated polymerase and capping enzymes (Grimes et al., 1998) A study utilising several monoclonal antibodies to VP7 indicated that VP7 (which includes a RGD motif) is not exposed at the surface of the complete virus particle (Hutchinson, 1999).

Although the *Orbivirus* particles are usually thought to comprise of just the genomic RNA and proteins VP1 to VP7, there are reports of NS2 and RNA being associated with the particle, although this is dependent upon the purification procedures used (Burroughs et al., 1994; Mertens et al., 1987a).

Treatment of BTV particles with proteolytic enzymes, such as trypsin, or chymotrypsin results in cleavage of VP2 in the outer capsid and generates infectious subviral particles (ISVPs) (Marchi et al., 1995; Mertens et al., 1987a). Cleavage of VP2 to form ISVP also results in a loss of haemagglutination activity, thus implicating VP2 in this process (Mertens et al., 1987a). ISVP have ~1000 times enhanced infectivity for adult *Culicoides* and for *Culicoides sonorensis* cells (KC cells (Wechsler et al., 1991)) compared to unmodified virus particles, but largely unchanged infectivity for BHK cells, suggesting that proteases (for example in *Culicoides* saliva) may play an important role in the BTV transmission cycle.

BTV core particles have different levels of infectivity when compared to intact virus particles containing the complete complement of VP2 and VP5. Cores are much less infectious (approximately $\times 10^{-4}$) for mammalian (e.g. BHK-21 cells) and in some cases are non-infectious (CHO cells), but are only 20-fold less infectious than intact virus particles for adult *Culicoides*, or a *Culicoides* cell line (KC cells) (Mertens et al., 1987a; Mertens et al., 1996). Cores can also be neutralised by antibodies to VP7 (Hutchinson, 1999). This indicates that in the absence of outer capsid proteins VP2 and VP5, cell attachment and penetration by the BTV core particle can be mediated by VP7. However, this presumably involves different binding sites / receptors and may even be mediated via a distinct cell entry pathway.

Removal of the core surface layer to leave just the VP3 capsid shell, or expression of VP3 by itself, generated sub-core or sub-core like particles (SCLP). Although subcores of BTV appear to be relatively unstable, they can be purified on CsCl gradients from equine encephalosis virus infected cells (EEV) (Mertens and Burroughs unpublished data). Orbivirus subcores contain the genomic dsRNAs and the polymerase /capping enzymes of the virus but are not thought to be infectious.

1.4: BTV Non-structural proteins

1.4.1: NS1

NS1 is a 64-kDa protein which represents the largest of the BTV non-structural proteins. The most conspicuous feature of NS1 is that it forms ‘tubules’ within the cytoplasm of infected cells. These are considered to be characteristic of orbivirus infection (Lecatsas, 1968; Murphy et al., 1971; Thomas and Miller, 1971; Tsai and Karstad, 1970).

BTV tubules vary in length between 52.3-1,000nm, with a helical structure composed of NS1 ‘ribbons’ (dimer-like structures) (Hewat et al., 1992a; Hewat et al., 1992b; Hewat et al., 1992c). BTV tubules have 21-22 ‘dimers’ per turn of the helix and have a diameter of approximately 52.3 nm (Hewat et al., 1992c). Interestingly, the NS1 tubules of different species of orbivirus appear to have different characteristics, including a different diameter, (Huismans and Els, 1979; Maree and Huismans, 1997; Moss and Nuttall, 1995; van Staden et al., 1998). The carboxy terminus, amino terminus and internal cysteine residues at positions 337 and 340 are all required for tubule formation (Monastyrskaya et al., 1994). The assembly of tubules is also observed when the protein is expressed in isolation (Urakawa and Roy, 1988).

NS1 is found predominantly in the cytoplasm of infected cells, with small amounts in the viral inclusion body (VIB) (Brookes et al., 1993; Eaton et al., 1988). It is highly conserved and is expressed early during BTV infection (Hwang et al., 1993; Whetter et al., 1990). It is also the most abundant viral protein expressed in BTV infected cells and has been found to be efficiently translated *in vitro* (Van Dijk and Huismans, 1988).

NS1 generates a strong antibody response in infected animals, although it does not contribute to neutralisation (Richards et al., 1988; Roy et al., 1990b). Antibodies to NS1 provide a marker for BTV infection (past or present) and are therefore considered as a potential target for diagnostic ELISAs (Anderson et al., 1993) or even DIVA assays. The carboxy terminal of NS1 is exposed on the outside of the tubule and contains an antigenic epitope (Monastyrskaya et al., 1995). The carboxy terminus can be modified by the addition of foreign peptides and proteins, demonstrating that NS1 is a useful delivery and presentation system for certain antigens (Mikhailov et al., 1996). Recombinant NS1 molecules, containing different epitopes were able to assemble into tubules, leading to the suggestion that it could be used for the concurrent delivery of multiple epitopes (Mikhailov et al., 1996). Modified NS1 tubules containing foreign epitopes, have also been used to induce both humoral and cytotoxic T cell (CTL) responses (Ghosh et al., 2002a; Ghosh et al., 2002b; Larke et al., 2005; Murphy and Roy, 2008).

The precise role of NS1 in BTV replication remains obscure, although it has been implicated in a number of different activities (Roy, 2008). Recent work suggests that NS1 may be involved in cellular pathogenicity (Owens et al., 2004). Disruption of tubule formation by the expression of a single-chain antibody to NS1 in a modified mammalian cell line, reduced the pathogenicity of the virus for these cells, and induced a switch from cell lysis to virus release by budding (Owens et al., 2004). This was accompanied by a 10-fold increase in virus titre in the tissue culture medium, and the resulting infection process appeared to resemble BTV infection of insect cells (Owens et al., 2004). In light of this work a theory was put forward to explain the difference in virus release between insect and mammalian cell systems (usually non-lytic/persistent, or lytic/cytopathic infections, respectively).

High levels of NS1 relative to NS3/NS3A resulted in a lytic form of virus release, while low NS1 levels relative to NS3/NS3A caused an increase in virus budding and reduced cell lysis (Owens et al., 2004). This work was based on preventing the formation of tubules, although it hasn't been proved that they are not simply a repository for 'used'

NS1. However, the inhibition of tubule formation and shift in the mechanism of viral release, suggest that it is the formation of NS1 into tubules itself, which alters cellular pathogenicity.

1.4.2: NS2

NS2 is a highly expressed protein in BTV infected cells and is a major component of the viral inclusion bodies (VIBs) that are characteristic of BTV and other orbivirus infections (Brookes et al., 1993). VIBs can be observed as early as four hours post infection (Brookes et al., 1993; Thomas et al., 1990). VIB are thought to be the major site of virus replication and assembly. Although ribosomes are excluded, the VIB is rich in ssRNA and all of the viral core proteins (Brookes et al., 1993; Thomas et al., 1990).

It has also been shown that NS2 binds to ssRNA via the N-terminus, and is specific for BTV (+) RNA (Fillmore et al., 2002; Lymeropoulos et al., 2006; Lymeropoulos et al., 2003; Theron and Nel, 1997; Zhao et al., 1994). Attempts to determine the precise manner in which NS2 interacts with ssRNA have lead to suggestions that this involves certain RNA secondary structures (loops), but with different structures for each segment, allowing discrimination between the different viral RNAs (Kar et al., 2007). It has also been shown that NS2 is the only orbivirus protein that is phosphorylated, a process that can be mediated by the cellular kinase CK2 (Huismans et al., 1987b; Modrof et al., 2005).

Structural studies on NS2 indicate an intermolecular dimer and suggest that it forms helical oligomers which have enhanced stability when interacting with RNA (Modrof et al., 2005; Ross-Smith, 2008). Phosphorylation reduces the affinity of NS2 for ssRNA and appears to increase the number of VIBs that form near the nucleus, in contrast to the diffuse NS2 staining pattern of the dephosphorylated protein (Modrof et al., 2005). The dynamic nature of NS2 is emphasised by its capability of hydrolysing ATP and GTP (Horscroft and Roy, 2000).

The labelling of VIB by antibodies to NS2 is largely restricted to the periphery, suggesting that it may be involved in some form of exchange between the VIB and the cytoplasm, depending on its phosphorylation state (Ross-Smith, 2008). It was hypothesised that it may be involved in transportation of BTV mRNAs from the VIB where they are synthesised, to ribosomes in the cytoplasm, via its interactions with the cellular cytoskeleton (Ross-Smith, 2008).

The importance of the role played by NS2 is also suggested by the expression of functional homologs by other members of the family *Reoviridae* (rotavirus and reovirus) (Horscroft and Roy, 2000; Taraporewala and Patton, 2004).

1.4.3: NS3/3A

The smallest BTV non-structural proteins are NS3 and NS3A, both of which are encoded by Seg-10 (Mertens et al., 1984). NS3A (25 kDa) lacks the 13 N-terminal amino acids of NS3 (28 kDa) and results from translation initiation at a secondary initiation codon. NS3/NS3A is the only BTV membrane and glycoprotein. It contains two hydrophobic transmembrane domains (amino acids 118-141 and amino acids 162-182) with the carboxy and amino termini located in the cytosol. Although the protein can be found un-glycosylated, there is an N-linked glycosylation site, amino acid 150 (asparagine) located between the two transmembrane domains (Bansal et al., 1998; Wu et al., 1992). As may be expected for a glycosylated protein containing hydrophobic transmembrane domains, NS3/3A appears to follow the secretory pathway. The protein is associated with the Golgi apparatus, smooth surfaced vesicles and the plasma membrane at points of virus release (Hyatt et al., 1991; Wu et al., 1992).

NS3 /NS3A are only expressed at very low levels in mammalian cells, but to a high level in insect cells, prompting speculation that NS3/NS3A are of critical importance in viral egress in insect systems (Guirakhoo et al., 1995; Hyatt et al., 1993; Van Dijk and Huismans, 1988). Yeast two-hybrid analysis revealed an interaction between the N-terminus of NS3 and p11, (the calpactin light chain) and between the C-terminus and the VP2 protein of virus particles (Beaton et al., 2002). The calpactin light chain is part of the annexin II complex, which is involved in cellular exocytosis, and could therefore provide a route to the cell surface for mature virions.

The advent of a reverse genetics system has allowed the roles of NS3/3A to be more precisely defined. Mutations in the C-terminal domain affecting the interaction with VP2 of the virus particle affected the virus release (resulting in a delayed cytopathic effect) but did not affect any protein function in virus replication (Celma and Roy, 2009). Two conserved late domain motifs (PPRY [amino acids 36-39], and PSAP, [amino acids 41-44]) have been identified in the N-terminal cytoplasmic domain of BTV NS3 (Wirblich et al., 2006). Research on other viruses containing late domains (e.g. retroviral gag proteins (Parent et al., 1995), Ebola virus VP40 (Harty et al., 2000) and Vesicular stomatitis virus matrix protein (Harty et al., 1999)), have indicated that the P(T/S)AP domain is responsible for recruiting Tsg101. Tsg101 is a component of the cellular

ESCRT-I complex, which is involved in formation of multi-vesicular bodies (MVB) that mediate protein sorting / degradation. When the impact of the late domain was investigated using reverse genetics, it was found that a mutant virus with the sequence GAAP instead of PSAP had delayed growth kinetics due to a defect in virus release by budding (Celma and Roy, 2009).

Most viruses that bind Tsg101 also contain a PPXY motif. This motif appears to be responsible for the recruitment of HECT containing host ubiquitin ligases e.g. Nedd4, and it appears that ubiquitination is important for viral release (Blot et al., 2004; Harty et al., 2000; Kikonyogo et al., 2001).

It is interesting to note that all of the previous viruses for which late domains have been identified are enveloped. Research has suggested that interaction of the viral late domain with cellular factors is responsible for budding of the virus from the cellular membrane. Although it is accepted that BTV readily buds from insect cells, release from mammalian cells appears to be predominantly via cell lysis. The work by Wirblich et al. (2006) demonstrated that the late domain motifs of BTV NS3 are capable of binding both insect and mammalian homologues of the host proteins Tsg101.

It has been suggested that the arginine, instead of proline, at the 'X' position of the late domain (PPXY) present on BTV NS3, influences interactions with host-cell components, resulting in different virus release phenotypes in insect and mammalian cells (Wirblich et al., 2006). There has also been a suggestion that NS3 can act in a manner reminiscent of viroporins, (small hydrophobic membrane pore-forming proteins) which disrupt cell membranes promoting virus release (Han and Harty, 2004). The suggestion that NS3/3A has an important role in disruption of the cell membrane is also supported by reassortant studies using AHSV which identified NS3/3A as a determinant of virus release and cell membrane permeability (Meiring et al., 2009).

Non-structural proteins are often highly conserved, at both the amino acid and nucleotide levels. Comparisons of the deduced BTV NS3/3A amino acid sequences to other orbiviruses have indicated that it is under negative (purifying) selection (Balasuriya et al., 2008; Hwang et al., 1992; van Staden and Huismans, 1991). A region of approximately 45 amino acids demonstrates over 40 % homology between *Culicoides* borne (AHSV, BTV) and tick borne (Broadhaven virus) orbiviruses (Moss et al., 1992; van Staden and Huismans, 1991). However, NS3/3A demonstrates considerable nucleotide and (outside of the semi-conserved region) amino acid variation (S. Maan, personal communication).

In light of its variability, Seg-10 of BTV and other orbiviruses has been the subject of extensive phylogenetic and molecular epidemiological analyses (Breard et al., 2007; Zientara et al., 1998). Seg-10 separates into a number of different groups that only partially reflect the geographic origins of individual virus isolates (topotypes), which may reflect the distribution of different vector species / populations (Breard et al., 2007; Pierce et al., 1998; Pritchard et al., 2004).

1.5: BTV replication

1.5.1: virus entry and uncoating

Infection of cells by BTV begins by adsorption of the virus particle to the cell surface via the outer capsid protein VP2 (Hassan and Roy, 1999). The cellular receptor in either insect or mammalian cells is unknown.

Several different forms of the virus particle can infect mammalian and insect cells (albeit with different efficiencies, see section 1.3.4) strongly suggesting that BTV enters cells by more than one mechanism. The RGD motif present in VP7 (amino acids 168-170) may be significant as these motifs are recognised by integrins (Grimes et al., 1998; Lewis and Grubman, 1990). Integrins are a family of α/β heterodimeric glycoproteins which act as adhesion molecules on the cell surface. Integrins are utilised by several viruses for cell attachment and entry, including foot and mouth disease virus (Jackson et al., 1997), adenovirus type 2 (Greber et al., 1993) and coxsackievirus A9 (Rovainen et al., 1994). It is possible that removal of the outer capsid proteins, at low pH exposes the RGD motif, allowing cores to bind and enter cells (Tan et al., 2001). However, previous work has indicated that cores interact with glycosaminoglycans, but not integrins (Hutchinson, 1999).

Different viruses can enter the cell using a diverse array of entry mechanisms. The simplest mechanism is by direct penetration or fusion of the plasma membrane in the case of enveloped viruses. Non-enveloped viruses more commonly enter cells via clathrin, caveolin or lipid raft mediated endocytosis, or macropinocytosis (Pelkmans and Helenius, 2003).

Clathrin mediated endocytosis has been widely reported as a path of viral entry among different virus families, e.g. Foot and Mouth Disease Virus (FMDV, Family *Picornaviridae*) and Bovine Viral Diarrhoea Virus (BVDV, Family *Flaviviridae*) and Semliki Forest Virus (Family *Togaviridae*) (Berryman et al., 2005; Lecot et al., 2005; Pelkmans and Helenius, 2003; Sieczkarski and Whittaker, 2005). The virus first binds to

the membrane which results in the formation of a pit. The pit becomes coated in clathrin and is endocytosed, resulting in a clathrin coated vesicle containing the virus. The vesicle fuses with an early endosome resulting in an acidic environment. It was reported by Eaton *et al* (1990) that BTV was observed in early endosomes. As described above, (section 1.3.4, Forms of the virus particle), a low pH environment results in removal of the BTV outer capsid proteins VP2 and VP5, releasing the core particle (Huismans *et al.*, 1987c). A low pH is required for the escape of many viruses from the endosome into the cytoplasm, often by inducing a structural rearrangement of a ‘fusion protein’ that can penetrate the endosome membrane (Gaudin *et al.*, 1995; Weissenhorn *et al.*, 2007). Fusion proteins have been widely studied and are found in many viruses e.g. the F protein of paramyxoviruses (Yanagi *et al.*, 2006). The ability of BTV-VP5 to insert into and destabilise membranes, suggests that its role is to release the core particle from the endosome into the host cell cytoplasm (Forzan and Roy, 2003; Hassan *et al.*, 2001).

1.5.2: Transcription

In common with many other viruses, the reoviruses replicate within the infected cell cytoplasm. However, in order to prevent the induction of the host cell anti-viral responses, the dsRNA genome itself cannot be exposed to the cell cytoplasm. As a result, reoviruses contain all of the necessary enzymes, including a helicase, polymerase and capping enzymes, within the core particle. The core becomes transcriptionally active on removal of the outer capsid proteins, before or during its release from the endosome (Van Dijk and Huismans, 1980). The BTV core associated polymerase synthesises full length mRNA copies of all ten of the genome segments within the host cell cytoplasm.

The transcription process must be deftly orchestrated, in order to simultaneously move each of the dsRNAs through different transcriptase complexes, within the finite space of the core (Gouet *et al.*, 1999). The transcription process is asymmetric such that only positive sense RNAs (mRNAs) are synthesised from the parental dsRNA. The viral mRNAs are capped by VP4 which forms part of the transcriptase complexes located at the 5-fold axes within the core. The nascent mRNAs are extruded from the core via ‘translocation portals’, also at the 5 fold axes (Figure 1.2 (Diprose *et al.*, 2001; Gouet *et al.*, 1999; Grimes *et al.*, 1998; Mertens and Diprose, 2004a)). Meanwhile, the substrates required for transcription (i.e. nucleotide triphosphates (NTPs) and the by-products of transcription enter and leave the particle by alternative channels (Diprose *et al.*, 2001). The dynamics of transcription remain unclear. Each particle is thought to contain a single copy of the dsRNA genome and, with the exception of Seg-5 and Seg-10, the molar ratios

of the resulting RNA match the expected amounts for a segment of a given size. These results suggest that each RNA is associated with a single TC. However, Seg-5 is transcribed in greater amounts than expected, whereas Seg-10 is transcribed less than expected (Van Dijk and Huismans, 1988). The ssRNAs synthesised from parental reovirus cores are capped, although it has been reported that progeny cores of mammalian orthoreovirus synthesise uncapped mRNAs. It has also been reported that cap dependent translation is inhibited in orthoreovirus infected cells resulting in shut-off of the translation of host cell, but not of the uncapped viral mRNAs (Eaton et al., 1990; Skup and Millward, 1980; Zarbl et al., 1980).

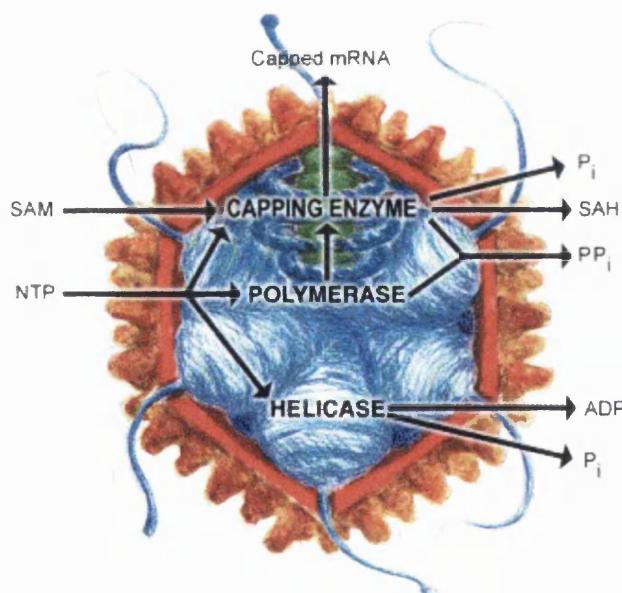


Figure 1. 2: A cartoon of a transcribing core particle.

Transcription complexes are located at the 5 fold axes and transcribe mRNAs from an individual segment. mRNAs are extruded through pores at the 5 fold axes whereas substrates and waste products enter/leave via alternative pores. (Mertens and Diprose, 2004a).

1.5.3: Translation

The translation of mRNAs involves the interaction of numerous proteins at multiple steps. The eukaryotic initiation factors (eIFs) are a suite of proteins which act at various points in translation, as scaffolding or RNA binding complexes. In the canonical model of translation, the eIF4F cap binding complex binds to the 5' cap of the mRNA.

Upon eIF4F binding, ribosomal subunits are recruited followed by ribosomal scanning and the initiation of translation. The eIF4F complex comprises eIF4E (which binds the 5' cap), eIF4A (an ATP-dependent helicase) and eIF4G (which interacts with both proteins and enhances the cap-eIF4E interaction).

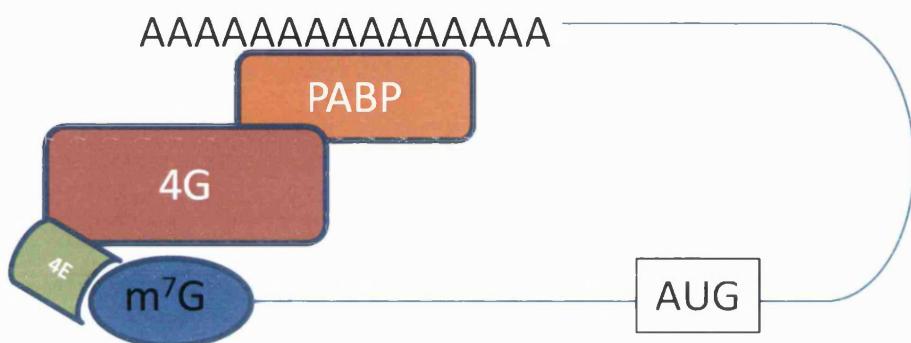


Figure 1. 3: A simplified cartoon of the canonical arrangement of eukaryotic initiation factors for the circularisation of polyadenylated mRNAs.
The eIF4G protein concurrently binds eIF4E (which is bound to the 5' cap) and PABP (which is bound to the poly(A) tail). This arrangement allows the circularisation of the mRNA that is required for efficient translation.

In eukaryotic cells, the polyadenylate (poly(A)) tail is bound by poly(A) binding protein, which in turn interacts with the eIF4F complex via eIF4G (Figure 1.3). The eIF3 protein stabilises these interactions by binding concurrently to numerous other proteins, and the 40S ribosomal subunit. The poly(A) interacting protein (PAIP) also binds to the eIF4F complex and is involved in translational control.

The mRNAs of BTV and other reoviruses have a 5' type 1 cap-structure identical to that of eukaryotic mRNAs and can therefore bind eIF4E. However, the 3' ends of viral mRNAs do not possess a poly(A) tail and are blocked by phosphorylation (Chow and Shatkin, 1975). Circularisation of the viral mRNAs during translation is therefore not possible using the cellular poly(A) binding protein (PABP) used for host cell mRNAs (Wells et al., 1998). It is possible that inverted repeats in the viral UTRs allow circularisation of the RNA in the absence of the poly(A) – PABP interaction (Markotter et al., 2004; Roy, 1989), although this has yet to be proven.

An alternative translation mechanism has been suggested for rotavirus (RV) (Piron et al., 1998), (Figure 1.4). Dimers of rotavirus non-structural protein NSP3 bind to

the termini of rotavirus mRNAs, which are also conserved (Deo et al., 2002; Piron et al., 1999; Poncet et al., 1993; Poncet et al., 1994). It is thought that NSP3 facilitates rotavirus mRNA translation by also binding eIF4G, resulting in circularisation of the RNA, effectively recreating the poly(A) – PABP – eIF4G interaction observed with poly(A) mRNAs (Piron et al., 1998; Vende et al., 2000).

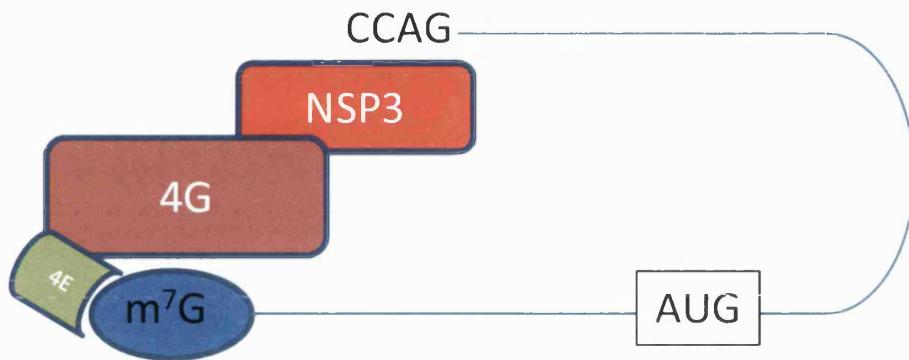


Figure 1. 4: The proposed model of rotaviral NSP3-dependent translation.

The eIF4E protein is bound both to the cap and to eIF4G in a manner reminiscent of eukaryotic translation initiation (Figure 1.3). In contrast to eukaryotic translation initiation, NSP3 replaces PABP on eIF4G and instead binds a 3' terminal sequence specific to rotaviral mRNAs. The result is a selective translation of rotaviral mRNAs and a decrease in the translation of polyadenylated mRNAs.

NSP3 binds to eIF4G in the same position as PABP (despite being structurally different), but with increased affinity, thus favouring the translation of rotaviral mRNAs (Groft and Burley, 2002; Piron et al., 1999; Piron et al., 1998), (Figure 1.4). NSP3 is also bound by the cellular protein RoXaN, a protein of unknown function (Vitour et al., 2004). The interaction of NSP3 with both eIF4G and RoXaN is required for the localisation of PABP to the nucleus during rotavirus infection (Harb et al., 2008).

However this model of rotavirus translation has been questioned. Knockdown of eIF4G using RNA silencing did not reduce viral protein synthesis (Montero et al., 2006). Knockdown of NSP3 resulted in an increase in viral RNA synthesis and allowed cellular protein synthesis to proceed (Montero et al., 2006).

Host-cell macromolecular synthesis is also shut-off in BTV infected mammalian cells, when viral synthesis is at its peak (Huismans, 1970b; Huismans, 1979). In contrast, the infection of insect cells or ovine $\gamma\delta$ T-cells results in a non-lytic persistent infection, and host cell protein synthesis is not thought to be shut-off (Huismans, 1971; Huismans, 1979; Takamatsu et al., 2003). BTV non-structural protein NS2 binds ssRNA and could represent a functional homologue of RV-NSP3. However, NSP3 has been shown to bind

to the conserved termini of RV mRNAs, while BTV NS2 does not bind the equivalent BTV mRNA sequences (Theron and Nel, 1997).

The ability of viral mRNAs to effectively compete with host cell mRNAs for translational machinery and substrates is fundamental to their translation and therefore the success of virus replication. Viruses have adopted a variety of ways, such as the proposed mechanism involving RV NSP3, to take over host cell translational machinery, which has evolved to work most efficiently with host cell mRNAs. Alternative strategies for biasing translation towards viral protein synthesis often involve cleavage or relocalisation of host cell translation factors (Alvarez et al., 2006; Belsham et al., 2000; Blakqori et al., 2009; Devaney et al., 1988; Kuyumcu-Martinez et al., 2004; Kuyumcu-Martinez et al., 2002). However, after it has disabled cellular translation machinery, the virus must also have a mechanism by which it can escape translational inhibition, for example translation from an internal ribosomal entry site (IRES) as observed in the picornaviruses (Belsham, 2000).

1.5.4: Assembly and exit

Progeny BTV core-particles are assembled within VIB before the outer capsid proteins are added at the VIB periphery, as they are released into the cytoplasm (Brookes et al., 1993; Gould et al., 1988). The process of particle assembly has largely been deduced from structural studies and the production of CLP and VLP by expression of BTV proteins from recombinant baculoviruses (French and Roy, 1990; Loudon and Roy, 1991; Roy et al., 1997). It is thought that VP3 initially forms dish shaped decamers (see section 1.3.2) which acts as a ‘scaffold’ for attachment of the replicase complexes, Twelve of these decamers also assemble to form the subcore-shell, (Grimes et al., 1998). Trimers of the outer core protein VP7, then decorate the sub-core, probably starting at the 3-fold, then step wise towards the 5 fold axis. The selection and packaging mechanisms for the genomic RNA are unknown.

Intracellular transport of the progeny virus particles is thought to be mediated by NS3/3A, resulting in their movement to the cell surface where virus release occurs (Beaton et al., 2002; Celma and Roy, 2009; Hyatt et al., 1993). It has been proposed that interactions of the virus with the host cell cytoskeleton, the p11 calpactin protein and endosomal sorting complex required for transport (ESCRT) pathway are all required for the release of the virus particles (Beaton et al., 2002; Bhattacharya et al., 2007; Eaton et al., 1988; Wirblich et al., 2006).

Yeast-two-hybrid and reverse genetics studies have indicated that NS3 acts as a bridge between the cellular export machinery (NS3 amino terminal and p11) and the virus (NS3 carboxy terminal and VP2) (Beaton et al., 2002; Celma and Roy, 2009). The interaction with the Tsg101 component of the ESCRT-1 pathway is thought to be mediated by NS3/3A via interactions with the proline-rich ‘late domain’ of NS3/3A (Celma and Roy, 2009; Wirblich et al., 2006).

It has been suggested that the BTV NS3/3A protein is cytotoxic. NS3/3A locates to the point of particle release and may form pores in the plasma membrane acting as a ‘viroporin’ (Han and Harty, 2004). AHSV NS3/3A has been shown to increase the permeability of the plasma membrane (Hyatt et al., 1991; Meiring et al., 2009; van Niekerk et al., 2001). However, NS3/3A is expressed relatively poorly in mammalian cells, leading to suggestions that its importance in virus release from mammalian cells is limited. In contrast, the high levels of NS3/3A expression observed may indicate that NS3/3A mediates budding/non-lytic release of progeny virus particles from persistently infected insect cells (Guirakhoo et al., 1995; Hyatt et al., 1993). Once released, the progeny particles are able to infect new cells or ‘superinfect’ the same cell (Figure 1.5). Superinfection then results in further amplification of the replication process (Hyatt et al., 1989).

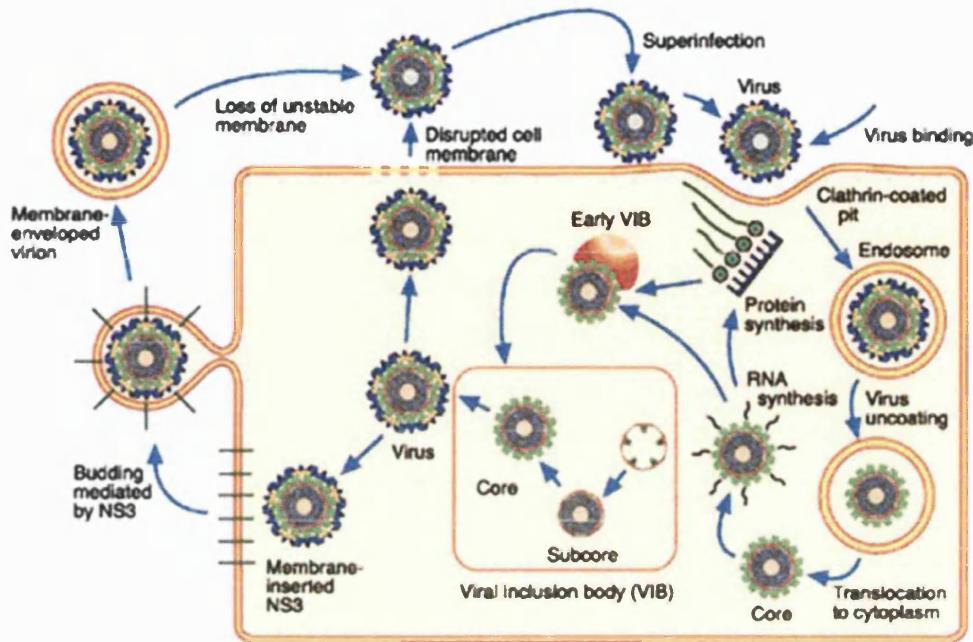


Figure 1. 5: A cartoon of the BTV replication cycle.
Reproduced with the permission of Professor Peter Mertens.

1.6: BTV Distribution and Transmission

1.6.1: Distribution

“Whoever would study medicine aright must learn of the following subjects. First, he must consider the effect of each of the seasons of the year, and the differences between them. Secondly, he must study the warm and cold winds, both those which are common to every country, and those peculiar to a particular locality”.

HIPPOCRATES, ON AIRS, WATERS & PLACES, C. 430 BC.

Antarctica remains the only continent where BTV has yet to be isolated (Gibbs and Greiner, 1994). Its distribution was historically limited to approximately 35 °S and 40 °N, with the northern limit extending to 50 °N in western America and China (Dulac et al., 1989; Gibbs and Greiner, 1994; Sellers and Mellor, 1993). This reflects the presence of competent vectors in regions that are sufficiently warm to allow virogenesis (Sellers and Mellor, 1993). In some areas the BTVs present remain relatively stable, for example the continuous presence of serotypes 2, 10, 11 13 and 17 in the USA. This may reflect a level of co-evolution between the local *Culicoides* midges and the virus (Gibbs et al., 1992). The regions in which BTV has been found can be divided into ‘endemic’ (where BTV is indefinitely present), ‘epidemic’, (where epidemics occur routinely every few years), and ‘incursion’ (where outbreaks occur only occasionally). Until 1998 the EU

represented an incursion zone (Gibbs and Greiner, 1994; Mellor and Wittmann, 2002). Occasional BT had occurred in the fringes of the Mediterranean basin, e.g. Portugal and Spain in 1956 (Manso-Ribeiro et al., 1957), western Turkey in 1977, and Lesbos in 1979 (Boorman, 1986; Mellor et al., 1985; Taylor and Mellor, 1994).

However, the distribution of BTV has changed dramatically in recent years. Seven new types (1, 3, 5, 6, 14, 19 and 22) have been discovered in the USA, reflecting northwards movement from central America or the Caribbean, or novel introductions from elsewhere in the world (P. Mertens and S. Maan, personal communication). Since 1998, BTVs representing serotypes 1, 2, 4, 6, 8, 9, 11, 16 and 25 have all invaded and persisted in Europe. This situation contrasts to previous outbreaks which tended to consist of ephemeral outbreaks involving a single serotype e.g. Cyprus in 1943 (Gambles, 1949). Nucleotide sequencing (primarily of Seg-2 - encoding outer capsid protein VP2) has become routine for the molecular characterisation of BTVs (Maan et al., 2008; Maan et al., 2007a; Maan, 2009). The use of these sequence data for 'molecular epidemiology' studies has greatly increased the resolution achieved in investigations concerning the presence and origins of novel BTV strains (Maan et al., 2007a; Purse et al., 2005). These studies have shown that more than one strain of BTV serotypes 1, 2, 4 and 9 have entered the Mediterranean basin since 1998. Reassortant viruses have also been detected in many of the Mediterranean outbreaks by full genome sequencing, suggesting that this can provide a better understanding of virus movements and evolution in the region (Batten et al., 2008b; Monaco et al., 2006b).

1.6.2: The current BTV Epizootic – 1998 onwards

Several different BTV strains have entered Europe from North Africa, and the Middle East. Live attenuated BTV vaccine strains have also been widely used in Italy and the western Mediterranean Islands. Importantly, these viruses were able to overwinter in the newly affected regions. Early research suggested that the northwards movement of BTV into Europe is related to global warming and the increased distribution of the Afro-Asiatic vector *C. imicola* in the region (Mellor and Wittmann, 2002; Purse et al., 2005; Wittmann et al., 2001). However, outbreaks of BTV-9 and BTV-16 have also occurred in the Balkan region (in 2002) in the absence of *C. imicola*, demonstrating the involvement of novel vectors (Listes et al., 2009; Omeragic et al., 2009).

A press release on the 17th August 2006 stated that BTV was suspected on several farms in the southern regions of The Netherlands, further north in Europe than had ever previously been recorded (Braakman, 2006). The presence of BTV was confirmed the

following day by Dutch laboratories and subsequently by the Institute for Animal Health, Pirbright; using antibody competitive ELISA, conventional RT-PCR assays, and the real-time RT-PCR assay developed as part of this project (Shaw et al., 2007). The real-time RT-PCR assay is described in Chapter 3 and Appendix III.

The virus was discovered on other farms in The Netherlands, and on farms in Belgium, Germany and France, indicating that transmission was occurring and widespread in the region (Elbers et al., 2008a). The old world vector (*C. imicola*) is absent at this latitude, indicating that a novel vector was involved in the virus transmission in the region (Mehlhorn et al., 2007). *Culicoides* species belonging to the *C. obsolitus* and *C. pulicaris* complexes are abundant across the whole of central and northern Europe and are considered likely to contribute to BTV transmission. Subsequent research has suggested that *C. dewulfi*, a member of the *C. obsoletus* complex may be particularly significant in this respect (Carpenter et al., 2006). The Netherlands isolate was identified as BTV serotype 8 by conventional RT-PCR and subsequently confirmed by Seg-2 sequence analysis (Maan et al 2007a, 2008). BTV-8 had not previously been isolated in Europe. Phylogenetic analyses indicated that the most closely related strains of BTV-8 existed in central Africa (Maan et al., 2008). It is unclear how this virus entered Europe, although it has been suggested that an imported zoo animal, or accidental importation of an infected midge are the most likely scenarios (Mintiens et al., 2008).

In 2007, BTV-8 re-emerged in many areas of northern Europe, having overwintered from the previous year. In the late summer and autumn of 2007 BTV-8 spread extensively across Europe, including the UK, Denmark, Poland, Switzerland and the Czech Republic. During the second year, the BTV-8 outbreak was extremely severe with widespread infection, and high levels of morbidity and mortality (up to 50% case fatalities in infected sheep (Elbers et al., 2008b; Meroc et al., 2009; Szmaragd et al., 2007).

In addition to BTV-8, a novel strain of BTV-1 spread up through Spain, Portugal, southern France and then northern France during 2007-2009, also posing a further threat to the UK.

During the late stages of 2008 / early 2009, two vaccine strains of BTV-6 and BTV-11 were also detected in the Netherlands, Belgium and Germany. There was evidence for reassortment of these strains with the widespread BTV-8 already in the region (K. Nomikou, personal communication). The arrival of the viruses has been

attributed to the illegal use of the South African live attenuated vaccines in the region, although this has not been confirmed (P. Mertens, personal communication).

1.6.3: Transmission

1.6.3.1: *Culicoides*

It had long been known that hematophagous insects (those where a blood-meal is required for egg maturation) are responsible for the transmission of BTV. However, BTV transmission to sheep by adult female of the biting midge *Culicoides imicola* (*pallidipennis*) (Culicoides, Diptera: Ceratopogonidae) was only demonstrated in 1943 (Du Toit, 1944). *C. imicola* is the primary 'old world' *Culicoides* species responsible for outbreaks of BT and other orbivirus diseases, including African horsesickness (AHS). *C. sonorensis* (formerly *C. variipennis*) was identified as a major vector for BTV in the USA (Price and Hardy, 1954).

As an arbovirus, BTV replicates in the arthropod vector, as well as the mammalian host. This was confirmed using electron microscopy by Bowne and Jones (1966), who visualised replicating BTV within the salivary glands of infected midges. The passage of BTV through the *Culicoides* vectors can be restricted by a number of 'barriers' each of which must be overcome in order for transmission to occur. The first of these, 'the 'mesenteron infection barrier' (MIB) occurs in the mid-gut of the midge after ingestion of a viraemic blood meal. However, if the cells lining the midges do become infected, a second barrier, the 'mesenteron escape barrier' (MEB) can restrict or prevent release of the progeny virus into the haemoceol of the insect (Fu et al., 1999). A further 'dissemination barrier' (DB), was first observed in *C. sonorensis* by (Fu et al., 1999), that restricts infection of secondary organs. In order for transmission of the virus to occur during subsequent blood meals, it must first infect and replicate in the midge salivary glands. There is no evidence for an infection or escape barrier within the salivary gland itself, and the saliva of infected midges can contain sufficient virus to result in up to 10 particles being 'injected' during a bloodfeed (Fu, 1995).

Recent Mediterranean and northern European epizootics of BTV (1998 onwards), have challenged ideas that BTV transmission is restricted to an individual vector species (*C. imicola* (old world) and *C. sonorensis* (new world)). Historically BTV had been largely dictated by the distribution of *C. imicola* which has recently become more widespread in the Mediterranean basin, due to increasing temperatures and climate change. However, BT outbreaks have occurred in regions of Europe beyond the northern distribution of *C. imicola*. In these areas, species of the *C. obsoletus* sensus latus (sl) and

possibly *C. pulicaris* complexes are thought to play a prominent role in transmission (Purse et al., 2005; Purse et al., 2006; Savini et al., 2005). Entomological studies performed in Bosnia and Herzegovina (after the BTV outbreaks in 2002) only identified *C. obsoletus* in light-trap catches (Omeragic et al., 2009). The outbreak of BTV-8 (and subsequently BTV-11 and BTV-6) in Northern Europe have helped to confirm that new vectors have contributed to the transmission of BTV.

The North European outbreak has prompted numerous entomological surveys in the region, showing that *C. obsoletus* sl is often the most abundant species complex (Balczun et al., 2009; Mehlhorn et al., 2009a; Mehlhorn et al., 2009b; Vorsprach et al., 2009). Members of the *C. obsoletus* sl complex, in particular *C. dewulfi*, can support replication of BTV (including BTV-8) in the laboratory (Carpenter et al., 2006; Carpenter et al., 2008a; Venter et al., 2005). However, the competence of the *C. pulicaris* complex has not been investigated to the same extent. This may be of significance for BTV epidemiology at higher altitudes, as *C. pulicaris* becomes the dominant species at increasing altitudes (Tschuor et al., 2009).

The vector competence of the *C. obsoletus* sl complex has important implications for the UK where these species are common. The spread of BTV-8 in 2007 demonstrated the potential for transmission in the UK, and laboratory analyses have shown that some populations of *C. obsoletus dewulfi* have similar levels of vector competence to *C. imicola* (Carpenter et al., 2006; Carpenter et al., 2008a).

In depth studies of *Culicoides* at the species level is difficult as the members of individual species belonging to a complex are morphologically identical. Recent development of molecular assays to discriminate between species, will facilitate these studies. These include PCR assays to differentiate *C. obsoletus* species by targeting differences between their internal transcribed spacer 1 (ITS-1) or mitochondrial cytochrome C oxidase I genes (Pages et al., 2009; Pages and Sarto, 2005; Schwenkenbecher et al., 2009; Stephan et al., 2009)

Orbivirus vector-transmission is a complex process involving interactions between both insect and viral proteins. It is therefore inevitably under the genetic control of both the insect and the virus. There is also considerable variation between individuals within a *Culicoides* population, regarding their ability to become infected and transmit BTV. Although some midge species or populations may be suboptimal for BTV transmission, the insect populations can be so great that transmission is still likely to

occur, simply because of the sheer number of insect bites (as demonstrated by the major Australian vector *C. brevitarsis* (Muller, 1987)).

1.6.3.2: Vertical transmission and direct transmission

Until recently it was believed that field strains of BTV were transmitted solely by the bites of *Culicoides* biting midge (Du Toit, 1944). However, some tissue culture adapted viruses have been shown to pass vertically by transplacental transmission, in the mammalian host (Parsonson et al., 1994; Richardson et al., 1985; Roeder et al., 1991). The North European BTV-8 field strain has also been shown to be efficiently transmitted transplacentally in both cattle and sheep (Menzies et al., 2008; Santman-Berends et al., 2009). BTV-8 has also been transmitted horizontally through the consumption of infected milk and, possibly, by eating the placenta of dams which have been infected with BTV (Menzies et al., 2008; Santman-Berends et al., 2009). Transplacental and oral transmission of BTV-8 was first observed in the field but has been reproduced experimentally (Backx et al., 2009). The aborted foetuses or newborn calves from infected dams were shown to have congenital defects such as hydrancephaly (Worwa et al., 2009; Wouda et al., 2009; Wouda et al., 2008).

1.7: Overwintering and persistence

The BTV transmission cycle is dependent on the presence and activity of vector species of *Culicoides* midges. It is still uncertain how BTV ‘overwinters’, surviving through periods of the year when *Culicoides* midges are absent. The transplacental transmission of BTV (as discussed above) represents one of several possible overwintering mechanisms (Santman-Berends et al., 2009; Wilson et al., 2008).

Although early observations report a link with erythrocytes, BTV has also been associated with blood monocytes (Stott et al., 1990; Whetter et al., 1989). Gamma-delta ($\gamma\delta$) T-cells have been proposed as a site of viral persistence in the mammalian host in an elegant system that would also allow overwintering of the virus in the absence of the *Culicoides* vector (Takamatsu et al., 2003).

It has also been suggested that overwintering can occur in persistently infected adult vector insects (Wilson et al., 2008). However, the detection viral RNA by RT-PCR in larvae collected from outbreak regions suggests that vertical transmission can also occur in at least some vector species, although efforts to isolate infectious virus from RT-PCR positive *Culicoides* larvae have consistently failed (White et al., 2005).

1.8: Pathogenesis and immunology

1.8.1: Infection of the mammalian host

Bluetongue is often regarded as a disease primarily of sheep and deer, although it is thought that it is able to infect all ruminants, as well as camelid species (Hourigan and Klingsporn, 1975; MacLachlan, 1994b). There have also been cases of BTV infecting wild carnivores, dogs (through the application of contaminated vaccines), or lynx (by ingestion of infected meat (Akita et al., 1994; Alexander et al., 1994; Fernandez-Pacheco et al., 2008; Jauniaux et al., 2008; Levings et al., 1996; Meyer et al., 2009).

Not all breeds of sheep are affected equally. Indeed, the majority of BTV-infected ruminants in enzootic areas, show few if any clinical signs of BT. In situations where disease is observed, overt clinical signs are often restricted to the improved fine wool and mutton breeds of sheep, such as those commonly found in Europe (Darpel et al., 2007; Jeggo et al., 1983; Jeggo et al., 1987; Parsonson, 1990). The strain of virus involved can also affect the severity of disease observed. The occurrence of clinical disease, particularly in cattle, can also be a feature of new incursions of BTV into naïve populations of animals that are particularly susceptible to infection (Parsonson, 1990).

Clinical signs of BTV can occur in sheep from five days post infection (dpi), with clinical signs peaking at 7-10 dpi. Sheep infected with BTV develop a fever just before or concurrently with the clinical signs. The first signs of disease are often hyperaemia of the oral cavity and mucosal membranes, with conjunctivitis commonly apparent. One of the most severe clinical signs in sheep is the development of a facial oedema, which is accompanied by excessive salivation and nasal discharge. Respiratory distress can result from pulmonary infections or oedema in the lungs, with tachypnoea and hypopnoea in infected sheep. Corrinitis can cause sheep to be lame, resulting in unwillingness to stand, depression and anorexia. Epithelial necrosis of the nose and mouth can occur later during infection. The blue tongue and toticollis are rare symptoms and usually occur in fatal cases. (Erasmus, 1975; Gambles, 1949; Hardy and Price, 1952; Moulton, 1961; Parsonson, 1990; Pini, 1976; Thomas and Neitz, 1947).

Cattle can develop BTV viraemia, with little or no fever (Heidner et al., 1988; Hourigan and Klingsporn, 1975; Luedke et al., 1969; MacLachlan et al., 1990). Although cattle usually don't display severe clinical signs of BT, to the same extent as sheep, the recent outbreak of BTV in northern Europe has challenged the common perception that cattle are unaffected by BTV. There have been numerous reports of

clinical disease (including mortality) in cattle affected by the BTV-8 strain (Backx et al., 2007; Dal Pozzo et al., 2009a; Dal Pozzo et al., 2009b; Elbers et al., 2008a; Elbers et al., 2008b). The northern European strain of BTV-8 appears to be particularly severe and affected a small percentage of cattle in a similar manner to sheep, along with hyperaemia and ulceration of the teats (Elbers et al., 2008a). The similarity between cattle and sheep lends support to the suggestion that the pathogenesis of BTV in cattle is essentially the same as that in sheep (Barratt-Boyes and MacLachlan, 1995; MacLachlan, 1994b; MacLachlan et al., 1990). It has been suggested that cattle are less affected by BTV infection due to a different susceptibility of their pulmonary endothelium, and therefore a reduced impact upon the lungs (DeMaula et al., 2001; DeMaula et al., 2002; McLaughlin et al., 2003; Russell et al., 1996).

The BTV-8 strain which recently affected northern Europe has been also been found to cross the placenta and cause teratogenic effects in cattle, including congenital hydranencephaly (Vercauteren et al., 2008; Wouda et al., 2008). There has also been a report of 'blue eyes' in transplacentally infected calves born to BTV infected dams (Holzhauer and Vos, 2009).

After introduction into the mammalian host, BTV is thought to infect leukocytes which transport it to the regional draining lymph node, where it can be detected prior to viraemia. This suggests that the lymph node may be an important site for early replication of the virus (Lawman, 1979; Pini, 1976). The early infection of leukocytes, and replication in lymph nodes, leads to further dissemination of the virus to other lymph organs, despite a weak or undetectable viraemia (Barratt-Boyes and MacLachlan, 1994; Pini, 1976). Different cell types have been suggested as target for infection and dissemination, including neutrophils, monocytes, macrophages, lymphocytes and dendritic cells (Hemati et al., 2009; Lawman, 1979; Morrill and McConnell, 1985).

After virus dissemination a higher level viraemia is generated, and BTV infects and replicate in leukocytes, lymph organs, vascular endothelial cells, skin glands and fibroblasts and many other organs (Barratt-Boyes and MacLachlan, 1994; MacLachlan et al., 2009; MacLachlan et al., 1990; Mahrt and Osburn, 1986). The BTV viraemia in cattle blood can extend to over 100 dpi, although more usually it is under 60 dpi (Bonneau et al., 2002; Hourigan and Klingsporn, 1975; MacLachlan et al., 1994; Richards et al., 1988). Viraemia in sheep is also long, with virus most commonly isolated from 5 to 28 dpi (Bonneau et al., 2002; Nunamaker et al., 1992).

The infectivity of the virus at later timepoints during infection is uncertain, as the virus particles may be associated with erythrocytes through haemagglutination, but neutralised by host antibodies (Heidner et al., 1988; Luedke et al., 1969; MacLachlan et al., 1990). Indeed, there is evidence that BTV can be detected in blood for very long periods post infection by RT-PCR assays, even though it is not possible to detect infectivity in the later stages (MacLachlan et al., 1994). The association of BTV with erythrocytes during the late stage of infection, along with an extended viraemia has been suggested as a strategy to provide protection from the host immune response, while promoting the uptake by feeding *Culicoides* (Brewer and MacLachlan, 1994a; MacLachlan, 1994a; MacLachlan, 1995). However blood which is positive for RNA (even at time points >220 dpi) has been found to be non-infectious for *Culicoides* midges (Bonneau et al., 2002; MacLachlan et al., 1994; Tabachnick et al., 1996). The detection of viral RNAs does not by itself indicate that a RT-PCR positive animal is infectious and they may therefore be of limited epidemiological significance.

Cattle and African breeds of sheep are at least partially resistant to BTV and overt clinical signs are mild or unapparent (Erasmus, 2009). However, BTV can cause a severe haemorrhagic disease in susceptible populations of sheep. The severity of BT also varies with different strains of the virus, e.g. some of the South African vaccine strains are less virulent (Veronesi, 2009; Veronesi et al., 2005). It has been suggested that in some cases and in some locations the presence of BTV may go unnoticed due to a lack of clinical signs, and outbreaks may only be detected when the disease becomes apparent (Mellor, 1994), or by routine surveillance assays. Indeed, TOV was discovered due to routine pre-movement surveillance schemes in place due to the ongoing BTV-8 outbreak.

1.8.2: Immune response

Double stranded RNA is a potent stimulator of antiviral responses in mammalian cells. It has been shown that interferon is induced during BTV infection (Fulton and Pearson, 1982), although the retention of the genomic dsRNA inside a core and shut-off of host-cell protein synthesis may reduce both the induction and production of interferon as a result of BTV infection. In addition, the core of BTV has been shown to bind dsRNA, which may sequester any dsRNA that is released as a result of abortive particle assembly or degradation, keeping it away from the cellular dsRNA detection machinery (Diprose et al., 2002).

Double stranded RNA also activates RNA silencing responses. Although the genomic dsRNA remains inside a core, the secondary structure of the mRNAs could

potentially also induce RNA silencing. However, the VP6 helicase protein may represent an anti-silencing mechanism by unwinding secondary structure both in the mRNA target, and separating any ds siRNAs (P. Mertens and H. Attoui, personal communication).

Two groups of antibody are formed during infection with a particular BTV: serogroup specific and serotype specific (Huismans and Erasmus, 1981). The Serogroup-specific antibodies represent those that are formed against the outer core protein VP7, and other core and non-structural proteins (Huismans and Erasmus, 1981). The VP7 protein appears to be immunodominant and creates a strong antibody response in infected animals. These proteins are sufficiently conserved across the entire BTV species that similar antibodies are formed regardless of serotype, and their detection (particularly antibodies against VP7) therefore provide a basis of BTV-specific ELISA assays. It has been suggested that, by directing the immune response against a 'core' protein, the immune system may be diverted from the outer capsid and thus recognition of the complete virus particle (P. Mertens, personal communication).

The major outer capsid protein VP2 harbours the majority of the serotype-specific epitopes (Huismans and Erasmus, 1981). The extent of the humoral response to BTV infection (particularly the response to VP2 and the levels of neutralising antibodies) shows at least partial correlation with the level of protection against subsequent infection with the same serotype (Jeggo et al., 1984c). However, the diversity in VP2 antigenicity results in a lack of cross protection against infection by heterotypic serotypes of BTV (Huismans et al., 1987a; Jeggo et al., 1983). Although infection (or vaccination) of animals against a single serotype will protect animals only against infection with the same serotype, it has been found that the sequential infection/vaccination with at least 3 different serotypes resulted in protection against most other BTVs (Jeggo et al., 1983). However, if the viruses are used to infect the animal concurrently, the heterotypic antibody response is not elicited, possibly as a result of not all the viruses replicating efficiently (Jeggo et al., 1984b). The other outer capsid protein VP5 also contributes to the humoral response, possibly via its interactions with and effects on the conformation of VP2 (Mertens et al., 1989).

It has been established that BTV infected cattle and sheep develop a lymphopenia (Ellis et al., 1990). However, it has also been found that clearance of BTV from the blood of infected animals is not purely due to the humoral response (Richards et al., 1988). The transfer of thoracic duct lymphocytes derived from sheep that had been infected with BTV demonstrated that a level of protection was achieved with T cells alone (Jeggo et al.,

1984a; Jeggo et al., 1985). BTV proteins VP2, NS1 and, to a lesser extent, VP3, VP5, VP7 and NS3/3A are recognised as CTL immunogens (Andrew et al., 1995; Janardhana et al., 1999). The absence of NS1 in inactivated virus vaccines is conceivably a reason behind why CTLs are only induced by replicating virus (Jeggo and Wardley, 1982b). BTV-16 has gained notoriety due to the high pathogenicity of the vaccine strain used in Israel and Southern Europe (Monaco et al., 2006b). Interestingly, CTLs induced by BTV-16 in mice were poor at lysing cells infected with any of the BTV serotypes that were tested (BTV-1, 3, 4, 6, 10 and 16), and cells infected with BTV-16 were poorly recognised by BTV induced CTLs (Jeggo and Wardley, 1982a).

1.0: Diagnosis

1.9.1: Virological and serological approaches

Diagnosis of BTV has traditionally been based on virus isolation and subsequent characterisation of the virus isolate. This has previously involved intravenous injection of embryonated chicken eggs, followed by cell passage of homogenised hearts from any dead embryos in baby hamster kidney (BHK-21) cells. The virus was then identified by BTV specific ELISA, and could be serotyped in virus neutralisation assays using standardised antisera to each of the 24 BTV types.

BTV serology investigates the sera of potentially infected animal for the presence of BTV specific antibodies, and can be used to assess the prevalence of BTV in the field. Serological methods, such as a blocking ELISA (Anderson, 1984; Martyn et al., 1991) can be used to test any animal in an endemic or outbreak area, or can be used to monitor seroconversion in sentinel herds, as a way of detecting the incidence or introduction of BTV infection.

Originally serological tests were performed using agar gel immunodiffusion (AGID) assays (Jochim and Chow, 1969). Although the AGID is sensitive, it lacks specificity, takes a long time, lacks any level of quantification, and is unsuitable for high throughput of multiple samples (Ward et al., 1995). The AGID test has largely been superseded by superior assays, in particular by ELISA.

A competition ELISA can be used to detect BTV specific antibodies in test sera, by assessing their ability to bind to a standardised viral antigen, blocking subsequent interactions with a standard diagnostic antibody (Anderson, 1984; Anderson, 1985; Anderson et al., 1993). ELISA assays for the detection of BTV antibodies are now

available commercially and perform well (Batten et al., 2009a). The relevance of detecting antibodies as a screening method for infection becomes questionable, with the widespread use of vaccination. The replicating monovalent live vaccines and the inactivated vaccines (which contain semi-or un-purified virus) will both result in the production of antibodies to the non-structural and core proteins. A 'differentiation of infected from vaccinated animals (DIVA) test is therefore difficult, although attempts have been made to produce such an assay based upon infected animals producing more anti-NS3 antibodies than vaccinated animals (Barros et al., 2009).

1.9.2: Molecular approaches

Recently, a suite of molecular methods for detection of the BTV genome have been developed based on reverse transcription - polymerase chain reactions (RT-PCR) (Anthony et al., 2007; Aradaib et al., 2005; Aradaib et al., 1998; Aradaib et al., 2003; Billinis et al., 2001; Dangler et al., 1990; Jimenez-Clavero et al., 2006; Orru et al., 2004; Shad et al., 1997; Wade-Evans et al., 1990; Zientara et al., 2002). While the detection of the viral genome in a tissue or blood sample by RT-PCR does not conclusively demonstrate that the animal is currently infectious, it does show that it was recently infected. This can be of particular importance when screening products for international trade, e.g. semen, but it gives little indication of the epidemiological significance of that infection, (Tabachnick et al., 1996). In addition animals vaccinated with the inactivated BTV vaccines currently in use within Europe remain negative by RT-PCR, giving a basis for DIVA.

Many of the current RT-PCR assays use a 'nested' step and require agarose gel electrophoresis to identify the amplified cDNA. This makes the assay labour intensive and makes it necessary to open the reaction tubes post-amplification, increasing the possibility of contamination (and false positive results) unless strict procedures are adhered to. In contrast, real time RT-PCR assays use a closed tube format and detection methods, significantly increasing throughput and reducing contamination risks. There are currently five published real-time RT-PCR assays for the pan detection of BTV. Two assays are based upon Seg-1, two assays based upon Seg-5 and one assay targeting Seg-10 (Jimenez-Clavero et al., 2006; Orru et al., 2006; Shaw et al., 2007; Toussaint et al., 2007a). Most of the assays have been demonstrated to have broad sensitivity for the BTV serogroup. However, the Jimenez-Clavero *et al* (2006) Seg-5 group specific assay does not detect eastern viruses (Batten et al 2008) due to mismatches in the primer-probe

sequences, while the Seg-10 ‘molecular beacon’ assay has only been tested against a limited number of BTV isolates *in silico* (Jimenez-Clavero et al., 2006; Orru et al., 2006). Real-time RT-PCR assays are now also in use to detect specific BTV serotypes including BTV- 1, 2, 4, 6, 8, 9, 11, 16 and 25 (Batten et al., 2009b; Hoffmann et al., 2009b). Real-time RT-PCR has also been used to differentiate field and vaccine strains of BTV-2 and 9 in Italy (Elia et al., 2008; Orru et al., 2004). Interestingly, the Seg-10 assay remains the only one currently able to detect the recently identified TOV (Chaignat et al., 2009). Novel real-time RT-PCR assays have now also been developed for AHSV (Rodriguez-Sanchez et al., 2008), and EHDV (Wilson, 1999).

Increasingly, nucleotide sequencing is used for the characterisation of BTVs with numerous methods being developed and modified (Maan et al., 2007b; Potgieter et al., 2009). An important finding of recent sequencing studies is that although different topotypes of Seg-2 can exist within each serotype, the sequence of Seg-2 always ‘matches’ BTV serotype, (Maan et al., 2007a). This has allowed the development of molecular assays based upon RT-PCR for ‘typing’ of BTV (Mertens et al., 2007) with the result that unidentified BTVs can now be characterised within hours of receipt.

A further advantage of sequencing is that evolution and molecular epidemiology studies can be used as a basis for fine scale analysis of the movement and origins of specific virus isolates. Molecular epidemiology studies have been conducted using different segments of the genome, including Seg-2, Seg-3 and Seg-10 (Maan et al., 2008; Nomikou et al., 2009; Ozkul et al., 2009). However, in view of the potential importance of reassortment, there is an increasing move towards full genome sequencing for representative isolates from new BT outbreaks (Maan et al., 2008; Maan, 2009). Sequencing methodologies are continuously advancing and there are plans to routinely apply ‘deep’ sequencing technologies to the full genome analysis of BTV isolates in the near future (P. Mertens, M. Palmarini and P. Kellam, personal communication).

1.10: BT Control and Vaccination

1.10.1: livestock movements and vector control

BT is a non-contagious virus that is transmitted primarily by biting midges (Mellor, 1990). Consequently, the slaughter of infected animals is usually considered to be ineffective for control of BTV infection and transmission, the only exception being for rapid removal of an imported index case if it can be identified sufficiently early to prevent adult *Culicoides* taking an infectious blood meal. In the UK (and other countries

in Europe), strict movement bans form a major part of BTV control policies, to avoid long distance spread of the virus. Laboratory confirmation of infection, allows a zonal system is implemented. A 20 km control zone (CZ) is placed around any infected premises, within which animals may not be moved to or from the infected sites. A protection zone (PZ) of at least 100 km is placed around the CZ then a 50 km surveillance zone (SZ). Animals can be moved within the PZ and SZ and from the SZ to the PZ. Movements out of the PZ or SZ, e.g. to slaughter, are only allowed under license (DEFRA, 2008). Inevitably, such restrictions have a large socioeconomic impact upon farmers within these zones.

The control of the vectors using insecticides targeted at the adult stage is a method of control commonly employed against vector-borne pathogens. During recent outbreaks, Greece and Spain have used insecticides for adult midge control (S. Carpenter, personal communication). The impact of insecticides has been assessed for their ability to reduce the biting rates on ruminants (Carpenter et al., 2008b; Mehlhorn et al., 2008; Papadopoulos et al., 2009; Schmahl et al., 2009a; Schmahl et al., 2009b). However, *Culicoides* have a diverse range of breeding habitats and are therefore difficult to control fully. An additional way in which exposure to midge biting can be reduced, is by housing the animals. Combined with insect repellent or insecticide, such measures may reduce the chance that a midge will be able to bite and infect an animal, although the overall impact of these measures is still thought to be low.

1.10.2: Modified live virus vaccines (MLVs)

Several vaccines have been developed for BTV, most of which are based on live attenuated viruses. Attenuation of the monovalent ‘modified live virus’ (MLV) vaccines is achieved by multiple passage of the virus *in vitro*, adapting it to grow in tissue culture. This is intended to reduce or attenuate the ability of the virus to replicate and cause severe disease in the mammalian hosts, in theory, producing an anti-BTV immune response in vaccinated animals in the absence of clinical signs.

A level of success has been observed using MLVs and some have been found to induce protective immunity in vaccinated animals (Hunter and Modumo, 2001; Lacetera and Ronchi, 2004; Savini et al., 2007a). However, the live attenuated vaccines for BTV were largely developed in South Africa and subsequently tested upon local breeds of sheep (e.g. naturalised sub-breeds of Merino). However, the South African sheep that were used to test the virulence of these vaccines tend not to show overt clinical signs

even with wild-type field strains of BTV. In contrast to the naïve European sheep populations, they therefore have a high natural level of resistance to the disease.

Clinical signs associated with the use of live vaccines were first observed in 1955 with subsequent reports of teratogenic effects in foetuses, reflecting the ability of these vaccines to be transmitted transplacentally (Osburn et al., 1971; Schultz and Delay, 1955; Young and Cordy, 1964). When these vaccines are used to infect European fine-wool breeds of sheep, severe clinical signs are evident in conjunction with a sufficiently high viraemia for transmission (Veronesi et al., 2005). Susceptible sheep vaccinated with some of the MLVs developed severe BTV symptoms including fever, lameness and facial oedema. Indeed clinical signs associated with vaccination using the South African BTV-16 MLV, were sufficiently severe that its use has been prohibited.

Vaccine strains of BTV can also be transmitted by adult *Culicoides* and are circulating in the field in southern Europe (Ferrari et al., 2005; Monaco et al., 2006a). Some of the attenuated vaccines are multivalent, providing protection against two or more serotypes. However, this also provides an opportunity for genome segment reassortment and the possibility that novel virus strains will be generated (Stott et al., 1987). The BTV field strain of serotype 16 from Italy 2002 has acquired an NS1 gene (Seg-5) that is identical to that of the BTV-2 vaccine strain, providing direct evidence for genome segment reassortment in the field in Europe (Batten et al., 2008b).

The MLVs that are currently available are therefore considered to represent a poor option for use in Europe, where the objective is to eliminate the disease and disease transmission from a country or region (Alpar, 2009).

1.10.3: Inactivated vaccines

Non-replicating vaccines produced by inactivation of tissue culture grown viruses (using betapropiolactone, gamma irradiation or binary ethylamine) are inherently safer than MLVs (Campbell, 1985; Parker et al., 1975; Ramakrishnan et al., 2006; Savini et al., 2007b). These inactivated vaccines are immunogenic, safe and allow protection of animals from BTV challenge (Savini et al., 2009). Furthermore, commercially produced inactivated vaccines have been used successfully in controlling BTV in the recent European outbreaks (Mellor and Oura, 2008). However, two inoculations are required to achieve a protective response in cattle, and it is imperative that each batch of vaccine is successfully inactivated. Furthermore, the protection is thought to be relatively short lived compared to the MLVs, with a booster being required annually.

1.10.4: Next generation vaccines

Next generation vaccines are based upon the delivery of proteins to the vaccinee, which will generate an immune response that will protect the animal against challenge from a virus. The most complex manner in which to perform this has been production of VLPs from insect cells co-infected with baculoviruses expressing the inner (VP3 and VP7) and outer (VP2 and VP5) capsid-proteins that can assemble into particles that are reminiscent of virions (Belyaev and Roy, 1993; Loudon et al., 1991). There is also evidence that VLPs can illicit protection when challenged with both homologous and heterologous serotypes (Roy et al., 1994; Roy et al., 1992).

The BTV outer capsid protein VP2 is largely responsible for the neutralising antibody response in ruminants and has been shown to elicit protective immunity when injected into a sheep (Huismans et al., 1987a). VP2 (often in conjunction with other proteins) has therefore been produced in a variety of viral vectors such as baculoviruses, vaccinia virus, canarypox and capripox viruses, and as purified protein (Boone et al., 2007; Huismans et al., 1987a; Lobato et al., 1997; Roy et al., 1990b). The pox viruses can be used to infect the animal directly. A canarypox virus that co-expresses VP2 and VP5 induced protective levels of neutralising antibodies in vaccinated sheep (Boone et al., 2007), while a capripox virus expressing VP2, VP7, NS1 and NS3 provided partial protection (Perrin et al., 2007). Fragments of the VP2 protein of BTV have also been expressed in yeast and peanuts (Asthmaram et al., 2006; Asthmaram et al., 2007; Martyn et al., 1994), although challenge experiments have yet to be performed.

The outer core protein VP7 represents the group specific antigen for BTV and can confer at least partial protection against a virulent challenge strain ((Wade-Evans et al., 1996). Prospective vaccines have therefore been developed based on an assumption that a vaccine containing VP7 of one serotype would also raise a protective response in vaccinated animals against a heterotypic challenge virus (Wade-Evans et al., 1997; Wade-Evans et al., 1996).

The development of a reverse genetics system has brought forward the possibility of engineering live BTVs to act as vaccines (Roy et al., 2009). These authors propose to engineer a disabled infectious single cycle (DISC) vaccine strain that will be grown in a *trans*-complementing cell line (TCCL). These cells will provide a functional version of a specific viral gene product, which is disabled or missing from the ‘engineered’ virus. This will allow the virus to grow normally in the TCCL, and although it will generate virus capsid proteins as antigens, it will be unable to complete its replication cycle, or be

transmitted in the vaccinated host. One possible candidate is a virus with a defective VP6 (helicase) gene (Matsuo and Roy, 2009).

Another way in which to deliver antigens to cells is to vaccinate an animal with a DNA construct, e.g. a plasmid which is competent to express the protein intracellularly in mammalian systems. The antigens could then be processed and induce an immune response. Vaccines based upon this approach have also been considered for BTV (P. Mertens, personal communication), with some promising early antibody responses. An important limitation of using vaccines that are based on live attenuated virus strains, or partially purified and inactivated virus is our inability to distinguish between the antibody responses generated in response to vaccination vs. those generated in response to infection with BTV. The use of expressed or purified BTV structural proteins, as vaccine antigens, would create a subunit vaccine that would not elicit any antibodies to the non-structural proteins (e.g. to NS1). The signature antibody profiles of infected animals (including antibodies to NS1) would make it possible to determine whether the animals were infected or vaccinated. A recent DIVA test has been reported based upon utilising expressed NS3 in an indirect ELISA (Barros et al., 2009), although unpublished data suggest that it is not fully reliable (Philippe Pourquier, personal communication) .

1.11: Aims and Objectives

An enduring riddle in BTV research, and indeed that concerning many other arboviruses, is the contrasting cytopathologies observed in mammalian and insect cells. The molecular mechanism behind the severe, lytic infection observed in most (but not all) BTV infected mammalian cells, as opposed to the persistent and non-cytopathic infection of insect cells, remains to be elucidated.

The proposal by Owens *et al* (2004) that the relative levels of non-structural proteins NS1 and NS3/3A determine the outcome of infection (lytic vs. persistent) potentially offered an insight into the basis of cytopathogenicity. The general aims of this project were to explore this hypothesis, and the nature of interactions between infected mammalian cells and BTV non-structural proteins, as well as their roles in the outcome of infection (persistent or lytic), host-cell shut-off, development of CPE and the mechanism of induced cell death.

One of the primary goals was based around the NS3/3A : NS1 ratio hypothesis. This would be interrogated by expressing larger amounts of each protein intra-cellularly, from plasmids, in order to change their ratio. RNA silencing would also be used to

decrease translation of one or other of the relevant mRNAs, generated by the BTV core in infected cells.

In order to detect and quantify the virus, one of the first objectives of the project was the development of a novel real-time RT-PCR assay systems targeting BTV Seg-1.

Shut-off of host cell protein synthesis is observed in mammalian cells, but not in insect cells which become persistently infected with BTV. Previous work has shown that shut-off is at least partially controlled at the translational level (Stirling, 1996). Overall control and the relative translation efficiency of BTV and host-cell mRNAs were considered very likely to be of central importance in the development of persistent or lytic infections. The roles of the different BTV non-structural proteins, and mRNA sequences (particularly the terminal non-coding regions) in maintaining viral-protein expression during shut-off were therefore examined in uninfected and BTV infected mammalian cells.

It was considered possible that the growth and division of cells could affect the nature of the cellular response, and the outcome of BTV infection. This hypothesis was investigated by analysing the effects of BTV infection and the role of the non-structural proteins in mammalian cells cultured in the presence or absence of foetal bovine serum (to allow continued cell growth).

The specific aims of this project therefore included the following:

- Development of reagents and techniques to facilitate BTV research, including real-time RT-PCR assays (e.g. targeting Seg-1) to identify and quantify the virus.
- Expression of epitope tagged versions of NS1 and NS3/3A from plasmids (to alter the NS1:NS3/3A ratio during infection).
- Development of siRNA strategies, to knock down NS1 or NS3/3A levels in infected cells (to alter the NS1:NS3/3A ratio during infection).
- Investigate the role of BTV non-structural proteins in translation of BTV mRNAs during the replication cycle.
- Investigate the effect of BTV infection and the roles of BTV non-structural proteins in shut-off of host-cell protein synthesis.
- Investigate the effect of BTV infection (and particularly the non-structural proteins) on cytopathogenicity in mammalian cells.
- Investigate the effect of BTV infection (particularly the non-structural proteins) on the mammalian cell growth and cell cycle.

Overall, these studies were designed to further our understanding of BTV interactions with mammalian cells at a molecular level, and how such interactions determine the outcome of infection. It was expected that data obtained during this project would inform further studies, both in insects and in ruminants.

Chapter 2: Materials and Methods

2.1: Cell culture

2.1.1: Maintenance of cell cultures

Sterile cell culture methods and conditions were maintained throughout.

Baby hamster kidney (BHK) -21 cells were maintained in Glasgow's modified Eagle's medium (GMEM, Invitrogen) containing 2 mM L-glutamine and supplemented with 10 % foetal bovine serum (FBS), 5 % tryptose phosphate broth (TPB), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

Vero (African green monkey kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing HEPES and supplemented with 10 % FBS, 2 mM L-glutamine, 2.5 µg/ml Amphotericin B, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

Bovine pulmonary artery endothelial (BPAEC) cells were maintained in DMEM containing HEPES and supplemented with 15 % FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

Cells were routinely maintained in 175 cm² tissue culture flasks and passed when the cell sheet approached 90% confluence. Mammalian cells were removed from the bottom of the flask by rinsing the cell sheet three times with versine trypsin, followed by 10 minutes incubation at 37 °C. The released cells were aspirated in 10 ml of growth medium, transferred to a 25 ml universal tube and pelleted by low speed centrifugation (178 × g, 3 minutes). The supernatant was discarded and the cell pellet was resuspended in an appropriate volume of fresh growth media. A proportion of the suspension was added to a new flask containing appropriate fresh media, or diluted in growth media in preparation for seeding tissue culture plates.

For storage at -80 °C, cells were harvested as above, washed a second time in growth media, and the pellet re-suspended in 10% Dimethyl sulfoxide (DMSO)/90% FBS. The cell suspension was distributed into 1 ml aliquots which were then chilled to -80 °C

2.1.2: Seeding 6 well and 24 well tissue culture plates

Cells to be used for microscopy were seeded onto 13 mm diameter cover slips (Thermanox (VWR International) for electron microscopy, glass for confocal microscopy). The cover slips were placed in the bottom of the wells of 24 well tissue

culture plates and rinsed with 70 % ethanol. Excess ethanol was allowed to evaporate. Cells which had been freshly harvested were resuspended in 10 ml of growth media. A 1:50 or 1:80 dilution was prepared in fresh growth media for the preparation of wells with a confluence level of ~60 % (for virus infection and immunolabelling) or ~80-90% (for transfection) was achieved by the following day. 1 ml of the diluted cells was added to each well and the cells were incubated at 37 °C in 5 % CO₂. Antibiotic-free media was used if the cells were to be transfected using Lipofectamine 2000.

2.2: Viruses

Unless otherwise indicated, all experiments were performed using the BTV-16 vaccine strain (RSAvvvv/16) available from the Orbivirus collection, IAH Pirbright. Virus stocks were grown in BHK-21 cells. The supernatant of a confluent 175 cm² flask of uninfected BHK cells was discarded and 4 ml of a 1/10 dilution of stock virus seed was applied to the cell sheet. The virus was allowed to adsorb for 30 minutes at room temperature prior to the addition of 50 ml of serum free GMEM (containing antibiotics) and incubation at 37 °C in 5 % CO₂. The supernatant and cellular debris were harvested when 100 % CPE was achieved (~24-48 hours post infection).

The virus titre was determined by plaque assay. A ten-fold dilution series of the tissue culture harvest was prepared using unmodified GMEM. 200 µl was added to confluent BHK-21 cell sheets in six well plates and incubated for 30 minutes at room temperature to allow virus adsorption. The sample was discarded and the cells were covered with an Indubiose overlay (Table 2.1). The cells were incubated for 48 hours to allow plaques to appear. Plaques were visualised using methylene blue staining.

Component	Quantity
Indubiose	0.6 g
dH ₂ O	25 ml
2x Eagle's overlay medium	75 ml
Tryptose phosphate broth (TPB)	5 ml
Penicillin/streptomycin	1.5 ml
Foetal bovine serum	1.5 ml

Table 2. 1: Indubiose overlay.

Indubiose powder was melted in the dH₂O by microwaving for 1 minute in a glass bottle and then added to the remaining components.

2.3: Transfection of plasmids into mammalian cells

Transfections were performed using FuGene HD (Roche) according to the manufacturer's instructions. Transfection 'complexes' were generated by first diluting 2 µg of plasmid DNA in 100 µl of OptiMEM serum free media (SFM). 5 µl of FuGene HD was added to the diluted DNA, vortexed briefly to mix, and incubated for 15 minutes at room temperature. Transfections were performed in 24 well plates using 25 µl of the complexes added dropwise to 0.5 ml of media in the well. The plate was rocked to distribute the complexes before incubation at 37 °C in 5 % CO₂. The complexes were not removed.

2.4: Microscopy

2.4.1: Cell fixation

2.4.1a: 4% paraformaldehyde for light microscopy

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

2.4.1b: Methanol

The procedure for methanol fixation was as above but using ice cold methanol and fixing for five minutes at -20 °C prior to washing in PBS and storage at 4 °C in PBS.

2.4.1c: 2 % glutaraldehyde for electron microscopy

Cells grown on Thermanox coverslips and infected with RSAvvvv/16 for use in transmission electron microscopy were fixed by replacing the cell culture media with 2 % 'glutaraldehyde EM fix' and incubating at room temperature for two hours. The cells were further processed and analysed using transmission electron microscopy by the IAH Bioimaging department. Briefly, the samples were fixed in 1 % osmium tetroxide for ≥ 2 hours and then dehydrated by sequentially incubating the coverslip in 70 % ethanol (45 minutes), 90 % ethanol (15 minutes) and 100 % ethanol (2 × 15 minutes). The coverslips were infiltrated first with 1:1 propylene oxide: epoxy resin (30 minutes) and secondly with 100 % resin (1 hour). The coverslips were then allowed to polymerise in resin at 60

°C for approximately 20 hours before the Thermanox coverslip was removed. After a further 24 hours polymerisation, the cells were ready for sectioning.

2.5: RNA purification

2.5.1: Extraction of RNA from clarified tissue culture supernatants

The medium and debris of infected cell cultures showing 100 % CPE were harvested by first collecting the material into a 25 ml universal before clarification by centrifugation at $200 \times g$ for 10 minutes. RNA was extracted from clarified tissue culture supernatants using the QIAamp viral RNA kit (Qiagen) according to the manufacturer's conditions. 140 μ l of supernatant was added to 560 μ l buffer AVL and incubated at room temperature for 10 minutes to allow lysis, followed by the addition of 560 μ l of absolute ethanol. The lysed sample was applied to the spin column and the RNA bound to the membrane by centrifugation at $15,000 \times g$ for 1 minute. The column was washed sequentially with 500 μ l of buffers AW1 and AW2 (components of the kit), each time centrifuging the column and discarding the flow through. The RNA was eluted from the column in 40 μ l of RNase free water.

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

The supernatant was removed and discarded from cell sheets growing in 24 well plates. The cells were then lysed in 350 μ l of Buffer RLT (provided with the kit and containing added β -mercaptoethanol (β -ME)), collected in 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 μ l of 70 % ethanol was added to the lysate, mixed by pipetting, then the RNA was bound to the spin column membrane by centrifugation at $15,000 \times g$ for 30 seconds. The membrane was washed once with 700 μ l of RW1 buffer (provided with the kit), and twice with 500 μ l RPE buffer (provided with the kit and diluted with 4 volumes of 96-100 % ethanol as described in the kit instructions). The RNA was eluted from the column by two rounds of centrifugation, each time using 40 μ l of RNase-free water.

2.5.3: RNA extraction using TRIzol®

TRIzol® (Invitrogen) contains phenol and guanidinium isothiocyanate, which allows one-step sample lysis and purification of RNA. The input sample can vary but the steps downstream of the lysis step are identical.

The lysis step for purifying RNA from liquids involves adjusting the sample volume to 200 µl using nuclease free water, adding the sample to 1 ml of TRIzol®, and incubating for 5 minutes at room temperature. 200 µl of chloroform was then added to the samples which were vortexed for 15 seconds followed by incubation on ice for 10 minutes. The phases were separated into a lower red phase, an interphase, and an upper aqueous phase containing the RNA, by centrifugation at 4 °C (15, 000 × g for 15 minutes). The RNA was precipitated by transferring the aqueous phase to a new tube and adding an equal volume of isopropanol. The sample was centrifuged at 4 °C (15, 000 × g for 15 minutes) and the supernatant discarded. The RNA pellet was washed once using 70 % (v/v) ethanol before air-drying for 10 minutes. The pellet was then resuspended in 20 µl of nuclease free water. The RNA was either used immediately or stored at -80 °C.

2.5.4: Purification of *in vitro* synthesised RNA

RNAs were re-purified from *in vitro* transcription reactions, to separate them from, reaction components / by-products and template DNA, using the RNA clean-up protocol of the RNeasy mini kit (Qiagen). The reaction volume was first adjusted to 100 µl using RNase-free water, before adding 350 µl Buffer RLT (provided with the kit and containing 0.1 % β-ME). 250 µl of ethanol (96-100 %) was added to the sample and mixed. The RNA was bound to the spin column membrane by centrifugation at 15,000 g for 1 minute. The column was then washed twice using RPE buffer (provided with the kit and diluted with 4 volumes of 96-100 % ethanol as described in the kit instructions) by adding 500 µl of the buffer, centrifuging the column for 1 minute and discarding the flow-through. The RNA was eluted from the column by two rounds of centrifugation, each time using 40 µl of RNase-free water.

RNA was either used immediately or stored at -80 °C.

2.6: Denaturation of dsRNA

2.6.1: Chemical denaturation

2.5 μ l of total RNA was mixed with 2.5 μ l 20 mM methyl mercuric hydroxide (MMOH) and incubated at room temperature for 10 minutes prior to inactivation of the MMOH by addition of 1 μ l 0.7 M β -ME.

2.6.2: Heat denaturation

As an alternative to chemical denaturation, 6 μ l RNA samples were heated to 98 $^{\circ}$ C in a thermal cycler for five minutes before snap cooling on ice.

2.7: Reverse Transcription (RT)

Denatured RNA was mixed with 50 ng random hexamers (Promega) or 2 pmol of a gene specific primer (GSP) in a final volume of 12 μ l and heated to 70 $^{\circ}$ C for 5 minutes before snap cooling on ice. 8 μ l of a reverse transcription mastermix containing dNTPs (10 mM each), 2 μ l 0.1 M dithiothreitol (DTT) and 200 units of Molony Murine Leukaemia Virus reverse transcriptase (MMLV, Invitrogen) was added to the denatured RNA-hexamer/primer mix, incubated at room temperature for 10 minutes, before incubation at 37 $^{\circ}$ C for 1 hour. RT product was stored at -20 $^{\circ}$ C.

2.8: Polymerase Chain Reaction (PCR)

2.8.1: High fidelity PCR

2.8.1a: Amplification of Seg-5 and Seg-10 of RSAvvvv/16 using Platinum *Taq* High Fidelity (Invitrogen):

As the nucleotide sequences of Seg-5 and Seg-10 had not been determined for BTV-16 (RSAvvvv/16), primers for their amplification were designed based upon an alignment of published sequences for other virus strains. The sense primer targeted the 5' UTR, as this is highly conserved across the entire BTV species. The antisense primer was designed to amplify the entire open reading frame, with the exception of the stop codon, which was excluded. This allowed the product to be cloned in frame with epitope tags using the pcDNA3.1 V5/HIS plasmid (see section 2.15).

PCR reactions contained 5 μ l of 10 \times High Fidelity PCR buffer (Invitrogen), 1 μ l dNTPs (10 mM each), 2 μ l 50 mM MgSO₄, 20 pmols each primer, 1 unit of Platinum *Taq*

High Fidelity polymerase, 2 μ l RT product and water to a final volume of 50 μ l. Amplification conditions are given in Table 2.2.

Temperature (°C)	Time	Cycles
94	2 min	1
94	30 sec	
60	30 sec	35
72	1 min per kb	
72	7	1
4	Hold	

Table 2. 2: The amplification conditions/thermal cycling used for *Platinum Taq* High Fidelity polymerase.

2.8.2: KOD polymerase PCR

For amplifications other than Seg-5 and Seg-10, for the creation of pcDNA3.1 V5/HIS constructs, all amplifications requiring high fidelity used KOD Hot Start polymerase (Novagen). KOD is a thermostable DNA polymerase derived from the bacterium *Thermococcus kodakaraensis* with an extremely low mutation frequency of 0.0035 (0.013 for *Taq*, 0.0039 for *Pfu*) as determined using PCR products and a blue/white screening assay (Takagi et al., 1997).

KOD Hot Start reactions contained 5 μ l of 10 \times PCR buffer (Novagen), 3 μ l 25 mM MgSO₄, 5 μ l 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 μ l. 10 ng of plasmid template was used, or 2 μ l of RT product. Amplification conditions are provided in Table 2.3.

Temperature (°C)	Time	Cycles
95	2 min	1
95	20 sec	
55-60 ^a	10 sec	35
70	20 sec per kb	
70	10 min	1

Table 2. 3: The amplification conditions/thermal cycling used for KOD hotstart polymerase.

^a the annealing temperature varied according to the melting temperature of the primers being used for amplification.

The PCR products were visualised after 1.2% agarose gel electrophoresis (AGE) and staining with ethidium bromide.

2.8.3: Screening *E. coli* cultures for plasmid inserts using PCR

PCRs were used to screen bacterial cultures for the presence of the specific cDNA inserts. The 10 μ l PCR reactions (all components from Promega) contained 2 μ l 5 \times GoTaq green PCR buffer, 2 μ l 25 mM MgCl₂, 1 μ l 10mM dNTPs, 20 pmols of each primer and 0.1 units of GoTaq DNA polymerase. Where possible, the orientation of the insert was determined, using one primer site located on the plasmid and another appropriate primer targeting the insert. The template was added by dipping a pipette tip into the bacterial culture and then into the PCR reaction and the reactions run according to those given in Table 2.4.

Temperature (°C)	time	cycles
94	2 min	1
94	20 sec	
55	20 sec	35
72	1 min per kb	
72	10 min	1

Table 2. 4: The amplification conditions/thermal cycling used for screening bacterial colonies using GoTaq polymerase.

The PCR products were analysed by 1.2 % AGE, stained with ethidium bromide and visualised under UV illumination. The clones which generated a band of the appropriate / expected size were selected for further processing.

2.8.4: One-step RT-PCR amplification of BTV and EHDV Seg-1

One-step RT-PCRs were used for the synthesis and amplification of BTV cDNAs prior to direct sequencing. The ‘Qiagen one-step RT-PCR kit’ was used for reverse transcription and PCR amplification in a single reaction, according to the manufacturer’s instructions. Seg-1 sequences from representative isolates of BTV (Accession numbers: AY493686, L20445, L20446, L20447, L20508, AY154458, X12819, NC_006023), African horsesickness virus (AHSV, NC_007656) and Epizootic haemorrhagic disease virus (EHDV, AB186040) were obtained from the GenBank database. These sequences were aligned using MegAlign (DNAStar, Lasergene) and used to design redundant primer sets to amplify a 412 nucleotide fragment (nucleotides 19 – 430 according to GenBank accession number AY154458) of BTV or EHDV Seg-1. The RT-PCR reactions contained 10 µl of 5 × mastermix (Qiagen), 30 pmols of each primer, 10 mM of each dNTP, 2 µl of enzyme mix, and water to a final volume of 44 µl, before adding RNA that had been chemically denatured as described above. Amplification conditions are provided in Table 2.5.

Temperature (°C)	Time	Cycles
45	30 min	1
94	15 min	1
94	30 sec	
60	1 min	35
72	1 min	
72	7 min	1

Table 2. 5: The amplification conditions/thermal cycling used for the amplification of Seg-1 fragments using the Qiagen one-step RT-PCR kit.

The RT-PCR products were analysed by 1.2% AGE and stained with ethidium bromide. Bands of the correct size (viewed under UV light) were excised and purified from the gel slice before being sequenced.

2.9: Real-time RT-PCR

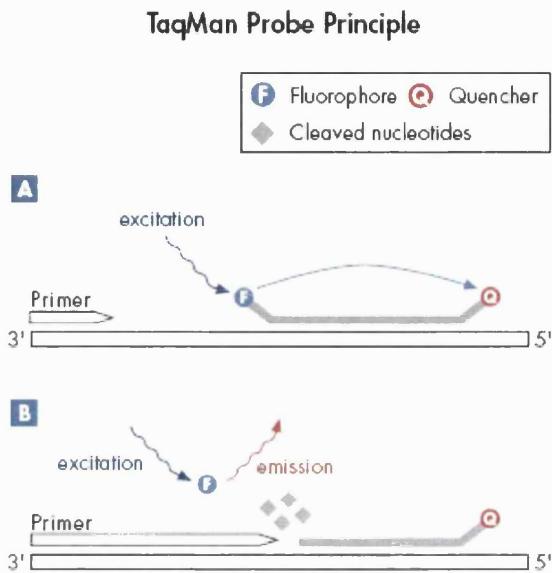


Figure 2. 1: The principle of TaqMan PCR.

The TaqMan PCR principle is based upon normal symmetric PCR. However, TaqMan probe-based PCR includes a dual-labelled probe in the reaction mixture. The probe is an oligonucleotide labelled at the 5' end with a fluorophore e.g. 6-Carboxyfluorescein (FAM) and the 3' end with a quencher e.g. black hole quencher (BHQ). Whilst the probe remains intact, the fluorophore fails to fluoresce upon excitation as the energy is transferred to the quencher by fluorescence resonance energy transfer (FRET). During the annealing step of the PCR cycle, the probe hybridises to the target strand between the probe sites. The 5' exonuclease activity of the polymerase cleaves off the fluorophore when the polymerase extends from the primer on the same strand as the probe. The energy is no longer quenched by FRET upon excitation leading to the release of energy from the fluorophore as fluorescence, which can be detected by the real-time thermal cycler.

TaqMan assays were designed according to standard guidelines

(<http://www.appliedbiosystems.com>).

1. Primers were designed to have a Tm of ~60 °C, while the probes were designed to have a Tm that is 10 °C higher.
2. The GC content of the probe should be 30-80 %. There should be more C residues than G.
3. Runs of identical nucleotides should be avoided in the probe, in particular for G residues.
4. Do not have a G residue at the 5' end of the probe.
5. Amplicon length should be 50-200 bp.

The probes designed for use in real-time assays, were labelled at their 5' termini with 6-carboxyfluorescein (FAM), and at their 3' termini with 6-carboxytetra-methylrhodamine (TAMRA) or black hole quencher 1 (BHQ-1).

The Superscript III Platinum *Taq* one-step qRT-PCR kit (Invitrogen) was used for real-time RT-PCR reactions. Half reactions (25 μ l as opposed to 50 μ l) were assembled by dividing the quantities suggested by the supplier and adjusting the final volume to 25 μ l using nuclease free water. The MgSO₄ was adjusted to 4 mM using the 50 mM stock provided with the kit

2.10: Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) was used for the separation of different nucleic acids on the basis of their individual molecular weights. The separated bands were stained with ethidium bromide in the gel (0.1 μ g/ml) and visualised under UV illumination.

All agarose gels were run at 3-5 V/cm in 1 \times TAE running buffer (National Diagnostics) until products were adequately separated. Agarose gels (1.2 % w/v) were prepared by melting electrophoresis grade agarose (Invitrogen) in 1 \times TAE buffer using the full-power setting of a microwave for 90 seconds. The agar was allowed to cool before adding ethidium bromide (0.1 μ g/ml), casting into the plastic 'tray' and allowing it to set.

For DNA fragments greater than 200 bp, Hyperladder I (Bioline) was run in an adjacent lane as a DNA marker. A 3.5 % agarose gel was used to give greater resolution of shorter fragments, e.g. real-time RT-PCR products (approximately 100 bp). The 25 bp ladder Hyperladder V (Bioline) was used to provide markers for short PCR products.

2.11: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.11.1 Sample preparation

Proteins from uninfected, transfected or infected cells were analysed by SDS PAGE in 10% gels. The media was removed from cells growing in 24 well plates and discarded. The cell sheets were rinsed once with PBS, and lysed in 200 μ l of lysis buffer C. The lysate was centrifuged at 15,000 \times g for 10 minutes at 4 °C to pellet cell debris. A 40 μ l sample of the lysate supernatant was added to 20 μ l of 3 \times PAGE sample buffer

(New England Biolabs) containing 12.5 mM DTT and boiled for 5 minutes for sample denaturation. A 10 % polyacrylamide gel with a 5 % stacking gel was loaded with the denatured sample and run under reducing conditions, using a method adapted from Laemmli (1970). Mini-gels (8 × 12 cm and 1 mm thick) were used for western blotting. The gels were run in Tris-glycine running buffer at 200 V until the dye front had just exited the resolving gel. One lane of the gel contained protein markers as guide to determine protein size and the efficiency of separation.

2.11.2: Autoradiography

SDS-PAGE gels were stained with destain containing Coomassie brilliant blue (Sigma), before destaining using destain and soaking the gel with Amplify™ fluorography reagent (GE Healthcare) for 30 minutes. The gel was dried onto Whatman 3MM blotting paper using a heated vacuum drier at 75 °C until the gel was dry and then exposed to X-ray film overnight at – 80 °C.

2.12: Western blotting

After separation by SDS-PAGE proteins were transferred by electrophoresis (1 hour at 100V / 400mA) from gels to nitrocellulose Immobilon-p membranes (Millipore) using transfer buffer (standard solutions, Appendix I). Non-specific binding of the primary antibodies was prevented by incubating the membranes overnight at 4 °C in blocking buffer (5 % (w/v) skimmed milk powder in TBS-Tween). The membranes were incubated with primary antibodies (anti-V5, Table 2.10, diluted 1:1000 into blocking buffer) for one hour at room temperature with agitation, washed for one hour in several changes of TBS-Tween, then incubated for one hour with a species-specific secondary antibody conjugated to horse radish peroxidase (HRP) (diluted 1:1000 in blocking buffer). The membrane was again washed several times in TBS-Tween then soaked in a 1:1 mixture of chemiluminescent reagents (ECL, GE Healthcare) for 1 minute. Excess reagent was removed before exposing the membrane to X-ray film.

2.13: Purification of DNA fragments

‘QIAquick PCR purification kits’ (Qiagen), or ‘illustra GFX PCR DNA and Gel band purification kits’ (GE Healthcare) were used to purify / recover DNA fragments

from enzymatic reactions or agarose gels (after AGE), according to the manufacturer's instructions. DNA fragments were eluted from the columns in 40 µl of nuclease free water. All purified DNAs were stored at -20 °C. DNA concentration and purity was assessed using the NanoDrop ND1000 spectrophotometer.

2.14: dATP tailing of blunt end PCR products

Blunt ended ligation of DNA by T4 DNA ligase is poor. The efficiency of the ligation reaction can be increased by using a vector with 3' thymidine (T) overhangs which creates a form of 'sticky end' ligation with the adenosine (A) overhangs added to the ends of PCR products by *Taq* DNA polymerase. 'TA' cloning takes advantage of the additional adenosine residue overhangs

For 'TA' cloning, an adenosine nucleotide is required at the end of blunt ended PCR products (those amplified using high fidelity DNA polymerases). Adenosine was added to the end of blunt PCR products by mixing 2 µl of purified PCR product in 1 µl of 10 × PCR buffer, 1 µl of 25 mM MgCl₂, 1 µl (5 units) of native *Taq* DNA polymerase (Promega), 1 µl of 1 mM dATP and 4 µl of nuclease free water. Tailing reactions were incubated at 70 °C for 30 minutes.

2.15: Cloning and constructs

A *NheI* restriction site was 'engineered' by RT-PCR at the 5' end of the cDNA amplicons of genome segments 5 and 10 by the addition of the restriction site in the primer, to allow transfer of the insert between selected vectors. For C terminal fusion constructs, the antisense PCR primer was designed to amplify the open reading frame, but eliminating the stop codon, allowing the protein to be cloned in-frame with a C terminal fusion protein. All clones and constructs were sequenced on both strands in order to verify that the correct insert was present and without unwanted mutations.

The pGEM T-easy system I (Promega) kit was used for the T-A subcloning of RT-PCR products.

The pcDNA3.1 V5/HIS vector (Invitrogen) is a mammalian expression vector for producing C-terminal fusion tags (V5 and 6HIS), which also utilises topoisomerase to increase ligation efficiency. This kit was used to generate pcDNA3.1 V5/HIS_NS3/3A

and pcDNA3.1 V5/HIS_NS1 which allow the expression of V5-tagged versions of NS3/3A and NS1 respectively, in mammalian cells (Figure 2.2 and described in Chapter 4).

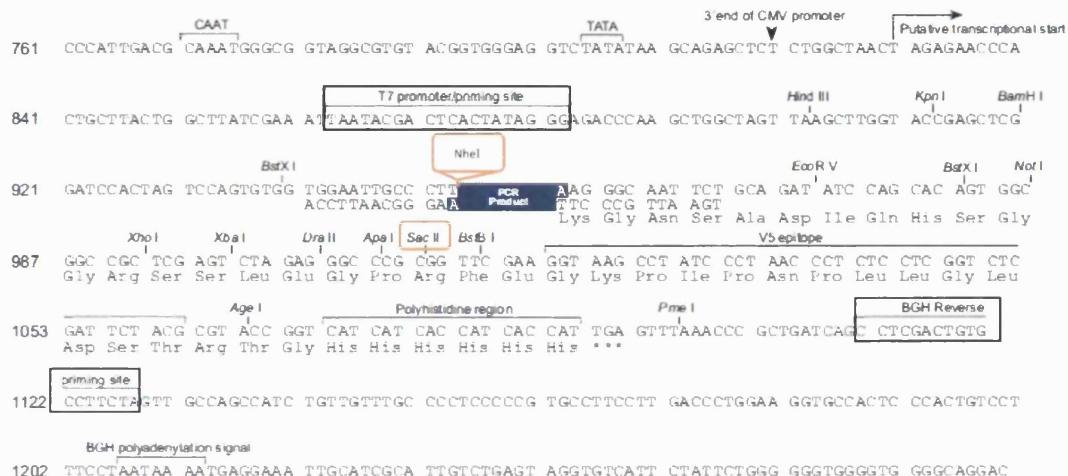


Figure 2. 2: The cloning site of pcDNA3.1 V5/HIS.

A RT-PCR product was amplified using *Platinum Taq* High Fidelity from reverse transcribed viral RNA and purified from agarose gels. The purified fragment was 'A-tailed' (section 2.14) and used in the cloning procedure according to the manufacturer's instructions (Invitrogen). The NheI restriction site was engineered at the 5' of the insert by incorporating the sequence in the sense primer. NheI and SacII were used during the production of pNS3/3A-EGFP. The footprints of the T7 and BGH primers used to sequence the plasmids pcDNA3.1 V5/HIS_NS3/3A and pcDNA3.1 V5/HIS_NS1 are highlighted.

The vector pEGFP-N1 (Clontech) is a mammalian expression vector for the transient expression of proteins fused to enhanced green fluorescent protein (EGFP, a modified version of the 26.9 kDa green fluorescent protein from the jellyfish *Aequorea victoria* (Shimomura et al., 1962)). The NS3/3A coding sequence was inserted 5' or 3' to the EGFP open reading frame to generate pEGFP-NS3/3A and pNS3/3A-EFGP respectively (described in Chapter 4).

2.16: Cloning reactions

2.16.1: Standard ligations

Ligation reactions contained of 20-100 ng of digested and purified plasmid vector, which had been dephosphorylated using shrimp alkaline phosphatase (SAP, Promega). The 'insert' was digested using the same restriction sites, then mixed with the vector at

molar ratios of vector to insert, of 1:1, 3:1 or 1:3, in reactions containing 1 µl 10 × ligation buffer (Promega), 1-3 units of T4 DNA ligase (Promega) and nuclease free water to a final volume of 10 µl. Ligation was allowed to proceed for 3 – 4 hours at approximately 16 °C, or overnight at 4 °C before transformation into competent DH5α *Escherichia coli* (*E. coli*).

2.16.2: cDNA cloning of Segment-10 into pcDNA3.1 V5/HIS TOPO (Invitrogen) and pEGFP-N1 (Clontech)

Platinum *Taq* High Fidelity polymerase was used to amplify the Seg-10 ORF cDNA (excluding the stop codon), using primers NheI NS3 and ‘no-stop BTV NS3 706-683R’ (Table 2.6). The resulting blunt ended PCR product was purified by AGE, then extracted from the gel, and a portion was ‘A-tailed’ using native *Taq* polymerase (Materials and Methods, section 2.14). 4 µl of the (unpurified) tailing reaction (see section 2.14) was combined with 1 µl each of the TOPO vector and salt solution, both provided with the expression kit. The ligation was incubated for 30 minutes at room temperature before transformation into the TOP10 chemically competent *E. coli* cells provided with the kit. The remaining PCR product was sequenced using the primers listed in Table 2.6.

Primer	Sequence	Purpose
NheI NS3 1-19F	GCTAGCGTTAAAAGTTCGCTGCC	Amplification of Seg-10 ORF
‘no-stop BTV NS3 706-683R’	GGTTAATGGYADTCRAAACCRCTC	Amplification of Seg-10 ORF
NS3 SEQ456-437R	GCCACRCTCATATCRCTYGA	Sequencing
NS3 SEQ268-287F	AGCGTTCTGTGATGATGTGA	Sequencing
T7	TAATACGACTCACTATAGGG	Sequencing ^a
BGH Reverse	TAGAAGGCACAGTCGAGG	Sequencing ^a
EGFP-N1 543-546F	GGTGGGAGGTCTATATAAGCAG	Sequencing ^b
EGFP-N1 1708-691R	GGTGAACAGCTCCTCGCC	Sequencing ^b
EGFP-N1 1342-1356F	CTGCTGGAGTTCTGTGACC	Sequencing ^c
EGFP-N1 1452-1429R	GTAAAACCTCTACAAATGTGGTAT	Sequencing ^c

Table 2. 6: The primers used to amplify and sequence the NS3/3A PCR product and plasmid insert.

The PCR primers that were used to generate the original plasmid insert were utilised as sequencing primers in conjunction with primers flanking the cloning site, and primers located within the insert sequence. Numbers refer to nucleotide position.

^aPrimers located on the pcDNA backbone and were used to sequence pcDNA3.1V5/HIS_NS3/3A.

^bPrimer located on the pEGFP-N1 backbone and was used to sequence pNS3/3A-EGFP.

^cPrimer located on the pEGFP-N1 backbone and was used to sequence pEGFP-NS3/3A.

The cloning reaction proved to be highly efficient for NS3/3A, producing a large number of colonies for analysis. Five colonies were amplified in LB broth for 'minipreps'. Sequencing of the resulting plasmids showed that three of the clones contained a full length insert, in the correct orientation and reading frame, with 100% sequence identity to the sequenced PCR product (Figure 4.2). A maxiprep of clone 33 was prepared, producing plasmid stock pcDNA3.1 V5/HIS_NS3/3A.

The NS3/3A gene insert was excised from plasmid pcDNA3.1 V5/HIS_NS3/3A, using the restriction enzymes NheI (a site introduced by PCR before cloning the gene into pcDNA) and SacII (Figure 2.2, Materials and Methods). The insert was gel purified and ligated into the pEGFP-N1 vector backbone, which had been digested with the same enzymes. The resulting plasmid, pEGFP-NS3/3A expresses NS3/3A with EGFP fused to the C terminus when transfected into mammalian cells (Figure 4.24).

Primers	BsrGI	NS3
(TGTACAAGCGGTCGAA <u>ATG</u> CTATCCGGGCTGATCC) (upstream) and NotI NS3r (GC GG CCGCTTAGGTTAATGGTAGTCGAAACC) (downstream) were used to amplify the NS3/3A open reading frame, incorporating a 5' BsrGI site and a 3' NotI site, using High Fidelity Platinum <i>Taq</i> polymerase with template cDNA produced by reverse transcribing viral RNA using MMLV, and A-tailed using <i>Taq</i> polymerase. 3 µl of the (un-purified) tailing reaction was combined with 1 µl pGEM T-easy vector, 5 µl 2 × rapid ligation buffer and 1-3 U T4 DNA ligase. Ligation reactions were incubated at 4-8 °C for 24-48 hours before transformation into competent <i>E. coli</i> . Positive plasmid minipreps were sequenced to ensure the authenticity of the insert.		

The insert was subsequently digested from pGEM T-easy using BsrGI and NotI and gel purified. The pEGFP-N1 backbone was similarly digested with BsrGI and NotI, and purified. A unique BsrGI site is located upstream of and near to the stop codon of EGFP, and a NotI site is located immediately after the stop codon in the plasmid 'pEGFP-N1' (Figure 2.3). The NS3/3A gene was inserted between these sites, complete with a stop codon at the downstream terminus of the NS3/3A gene. The insert was then ligated into the vector prior to transformation into DH5 α cells. This strategy generates a fused gene with an upstream EGFP sequence, and a downstream NS3/3A sequence (Figure 4.24).

A

GGGAGGT CTATATAAGCAGAGCTGGTTAGTGAACCGTCAGATCGCTAGCGCTACCGGA
Nhe I

CTCAGATCTCGAGCTCAAGCTCGAATTCTGCAGTCGACGGTACCGCGGGCCGGGATCC
Bgl II Xho I Sac I Hind III EcoRI Pst I Sal I/Acc I Kpn I Sac II Xba I/Sma I BamH I

ACCGGT CGCCACCATGGT GAGCAAGGGC 693
Age I

B

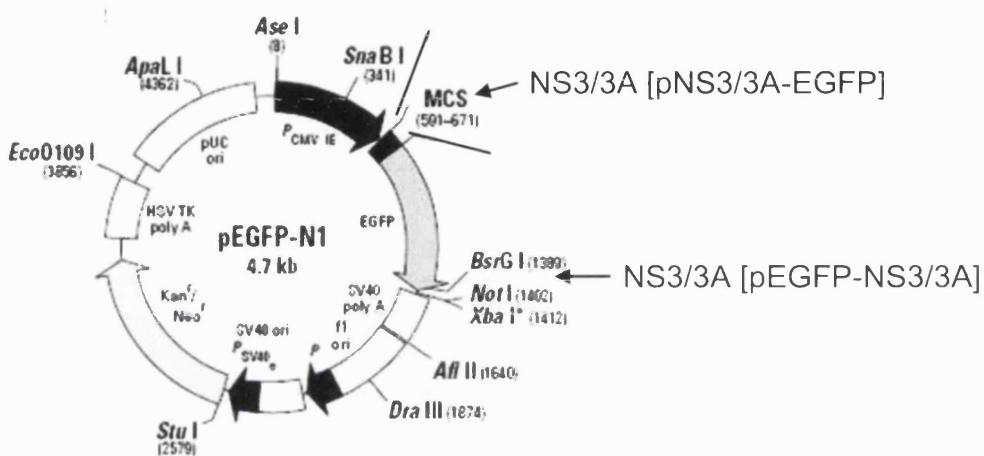


Figure 2. 3: The cloning strategy for assembly of pNS3/3A-EGFP and pEGFP-NS3/3A.

The NS3/3A insert for production of pNS3/3A-EGFP, was excised from pcDNA3.1V5/HIS NS3/3A using NheI and SacII before being ligated into the multiple cloning site of pEGFP-N1 (A).

To obtain the insert for pEGFP-NS3/3A the NS3/3A open reading frame was first amplified using primers containing BsrG1 and NotI restriction sites and subcloned into pGEM T-easy. The BsrG1 and NotI fragment was then ligated into the pEGFP-N1 which had been digested with the same enzymes (B).

2.16.3: cDNA cloning of Segment-5 into pcDNA3.1 V5/HIS TOPO

(Invitrogen) and pEGFP-N1

The high fidelity DNA polymerase Platinum *Taq* High Fidelity was used to amplify the open reading frame (ORF) of BTV-16 (RSAvvvv/16) Seg-5 encoding NS1, using viral RNA which had been reverse transcribed using MMLV (Materials and Methods, section 2.7). The upstream primer ‘Seg-5 NheI 1-20F’ targeted the 5’ conserved

untranslated region of Seg-5, and the downstream primer ‘no-stopNS1 1690-1667R’, targeted the 3’ end of the ORF but excluded the stop codon (Table 2.7).

After purification by AGE the amplicon, and topoisomerase ‘T-A’ cloning into pcDNA3.1 V5/HIS using the cloning kit (Invitrogen), the plasmid was transformed into competent TOP10 bacteria.

Primer	Sequence	Purpose
Seg-5 NheI 1-20F	GCTAGCGTTAAAAAAGTTCTCTAGTT	Amplification of Seg-5 ORF
no-stopNS1 1690-1667R'	TACTCCATCCACATCTGAGA CAT	Amplification of Seg-5 ORF
NS1 SEQ925-947F	GGC TTACCAATGATTATAGTGCAGGA	Sequencing
NS1 SEQ1185-1211F	GGGGATCATTACTATACAAACAAATTG	Sequencing
NS1 SEQ770-747R	ACTTCTTCAGCATAGCCGACTCTC	Sequencing
NS1 SEQ432-454F	TTA GAT TGG ATG ATT CDC TTT C	Sequencing
NS1 SEQ1322-1341F	GATGAAGGATGGCAGGAGGG	Sequencing
NS1 SEQ454-432R	GAAAGHGAATCATCCAATCTAA	Sequencing
NS1 SEQ1341-1322R	CCCTCCTGCCATCCTTCATC	Sequencing
T7	TAATACGACTCACTATAGGG	Sequencing ^a
BGH Reverse	TAGAAGGCACAGTCGAGG	Sequencing ^a

Table 2. 7: The primers used for the amplification and sequencing of Segment-5.

The PCR primers that were used to generate the original plasmid insert were utilised as sequencing primers in conjunction with primers flanking the cloning site, and primers located within the insert sequence. Numbers refer to nucleotide position.

^aPrimers located on the pcDNA backbone and were used to sequence pcDNA3.1V5/HIS_NS1, (see Materials and Methods, Figure 2.2).

The insert from plasmid ‘pcDNA3.1 V5/HIS_NS1’ was confirmed as ‘full-length’ and in the correct orientation by sequencing using primers listed in Table 2.7. It also confirmed that the insert was in-frame with the coding sequence for the epitope tags at the C terminus (Figure 4.8).

2.17: Preparation of chemically competent cells

The DH5 α strain of *E. coli* was used for the majority of cloning procedures. Chemically competent *E. coli* cells were made using a modified version of the calcium chloride method (Mandel and Higa, 1970). Briefly, 1 ml of an overnight culture was diluted in 10 ml of Luria-Bertani (LB) broth and incubated at 37 °C until the cells reached OD₆₀₀ 0.2-0.8. This culture was transferred to a flask with 50 ml of LB broth and grown until OD₆₀₀ 0.9. 200 ml of LB broth was added to the flask and incubated until the cells reached OD₆₀₀ 0.6. The cells were harvested by centrifugation (3000 \times g for 15 minutes), resuspended in 50 ml of fresh TF 1 buffer, and incubated on ice for 5 minutes.

The cells were harvested again and resuspended in 10 ml of ice cold TF buffer 2. The competent cells were aliquotted, snap frozen on ice and stored at – 80 °C.

Additional strains of bacteria were also tried in an effort to clone Seg-5. These strains included ABLE C (Stratagene), JM109 (Promega) and BL21 DE3 (Novagen).

2.18: Transformation of chemically competent cells with plasmids

Standard cloning protocols were followed according to Sambrook *et al* (1989). Competent cells were thawed on ice and 1-5 µl of the ligation reaction was pre-chilled in a fresh 1.5 ml microcentrifuge tube on ice. Once thawed, 50 or 100 µl of the competent cells were added to the ligation reaction (on ice). Transformations were incubated on ice for 30 minutes, heat shocked for exactly 45 seconds at the appropriate temperature (37 °C for DH5 α , BL21 DE3 and JM109 cells ; 42 °C for TOP10 and ABLE C cells) and immediately placed on ice for two minutes. 1 ml SOC medium (IAH Pirbright) was added and the tube incubated at 37 °C for 1 hour in a shaking incubator (200 rpm) before spreading 200 µl on pre-warmed LB agar plates containing the appropriate antibiotic(s).

For blue-white screening using the *lacZ* phenotype present in the pGEM T-easy vector, 90 µl bromo-chloro-indolyl-galactopyranoside (X-Gal, 20 mg/ml) was spread on the agar plate approximately 20 minutes prior to spreading the transformation reaction. After spreading, the plates were inverted and incubated at 37 °C overnight.

2.19: Clone amplification

2.19.1: Bacterial culture

All bacterial manipulations were performed using standard microbial techniques (Sambrook, 1989).

LB broth and LB agar supplied by the IAH central services were used for all bacterial cultures. LB agar was used for the isolation of clones, following transformation of bacteria or from a streaked glycerol stock. Ampicillin and kanamycin were used for selection of transformed colonies, depending on which antibiotic resistance was imparted by the plasmid used. Ampicillin was used at a final concentration of 100 µg/ml whereas kanamycin was used at a final concentration of 50 µg/ml media.

2.19.2: Microcultures

A high throughput ‘microculture’ method was used for screening colonies, where 100 µl of LB broth (containing appropriate antibiotics) was added to wells of a 96 well

PCR plate. Each well was inoculated with an individual colony, using a sterile pipette tip. These cultures were then covered with PCR foil and incubated overnight at either 30 °C or 37 °C, with shaking at 200 rpm. These cultures were then screened for relevant inserts using PCR (see section 2.8.3). Positive colonies were subsequently used to inoculate miniprep cultures.

2.19.3: Bacterial minipreps

Colonies were picked using a sterile pipette tip and grown overnight in 12 ml of LB broth at 37 °C, with shaking at 200 rpm. Glycerol stocks (150 µl sterile glycerol; 850 µl bacterial culture) were taken and stored at -80 °C. The remaining bacterial culture was harvested by centrifugation (3000 × g for 15 minutes) and plasmid DNA isolated using the Qiagen QIAprep miniprep kit according to the manufacturer's conditions, eluting plasmids in 50 µl of elution buffer (provided with the kit). Purified plasmids were stored at -20 °C.

2.19.4: Bacterial midipreps

Sufficient amounts of plasmid for a limited number of transfections were prepared using midiprep kits. The Qiagen 'HiSpeed midi kit' was used according to the manufacturer's instructions. The eluted plasmid was also ethanol precipitated in order to concentrate the DNA.

2.19.5: Bacterial maxipreps

Larger quantities of plasmid were isolated from 250 ml of bacterial culture using the 'Qiagen QIAprep maxiprep kit' according to the manufacturer's conditions with the exception that all centrifugation steps were 45 minutes at 1000 × g in a Sorvall SLA-600TC rotor. Precipitated plasmid was re-suspended in 500 µl of elution buffer (provided with the kit) and quantified using the NanoDrop ND1000 spectrophotometer.

2.20: Restriction digestion

Restriction digests were carried out as directed by the manufacturer (Table 2.6) in either 20 or 50 µl reactions. Typically a miniprep digest contained 2 µl of the appropriate 10 × restriction buffer, 5-10 units of each restriction enzyme, 0.2 µl of bovine serum albumin (BSA), 0.5 µg plasmid, and water to a final volume of 20 µl. Digests were incubated in a waterbath at the appropriate temperature for 1.5-2 hours.

Enzyme	Restriction Site	Manufacturer
NotI	GCGGCCGC	Promega
SacII	CCGCGG	Promega
NheI	GCTAGC	Promega
BsrGI	TGTACA	New England Biolabs

Table 2. 8: Details of the restriction enzymes used for cloning purposes.

Vectors that were restriction digested, were gel purified before being dephosphorylated for 15 minutes at 37 °C using SAP, (Promega) according to the manufacturer's conditions.

2.21: Nucleotide sequencing of PCR products and plasmids

2.21.1: Beckman sequencing

2.21.1a: Sequencing of PCR products

PCR products were sequenced in both orientations. Sequencing reactions contained DNA template (approximately 50 ng), 3.2 pmols primer, 8 µl Quickstart mastermix (Beckman CEQ) and nuclease free water to a final volume of 20 µl.

2.21.1b: Sequencing of plasmids

Approximately 200 ng of purified plasmid was added to nuclease free water to a final volume of 10 µl in a 0.2 ml reaction tube, and 'relaxed' by incubating for 1 minute at 96 °C in a thermal cycler. The template was then allowed to cool to room temperature before the addition of 3.2 pmols of the sequencing primer and 8 µl of Quickstart mastermix (Beckman).

Sequencing reactions of PCR products and plasmids were cycled as follows:

Temperature (°C)	Time	cycles
96	20 sec	
50	20 sec	30
60	4 min	
4	∞	

Table 2. 9: The thermal cycling profile used for the cycle sequencing of PCR products and plasmids using Beckman CEQ8000 chemistry.

Sequencing reactions were subsequently precipitated with ethanol in the presence of sodium acetate and ethylenediaminetetraacetic acid (EDTA) according to the Beckman protocol, re-suspended in 40 µl of sample loading solution (Beckman) and run on a Beckman sequencer (CEQ8000).

2.21.2: Applied biosystems sequencing

During the project the sequencing system at the IAH was changed from the Beckman system to the Applied Biosystems 'BigDye' chemistry. Applied Biosystems reactions were assembled as 1/16th (the Applied Biosystems mastermix was diluted using sequencing buffer) and contained 10 pmols of primer, DNA template according to Table 2.8, and 0.25 µl BigDye Terminator sequencing mix, then made up to a final volume of 10 µl with nuclease free water.

Fragment length (bp)	DNA template (ng)
100-200	5-20
200-500	10-40
500-1000	20-50
1000-2000	40-100
> 2000	50-150
plasmids	150-300

Table 2. 10: A table indicating the amounts of template to be used in sequencing reactions using the Applied Biosystems BigDye chemistry.

After thermal cycling (Table 2.9) the reactions were ethanol precipitated and resuspended in Hi-Di Formamide before running on an ABI 3730 Sequencer. Briefly, 2.5 μ l of 125 mM EDTA and 30 μ l of 100 % ethanol were added to the completed reaction, sealed and centrifuged at $3000 \times g$ for 30 minutes. The supernatant was discarded and the pellets washed twice with 70 % ethanol, each time centrifuging the sample at $1650 \times g$ for 15 minutes. The supernatant was discarded and the pellets dried under vacuum for 10 minutes. The pellets were then resuspended in the Hi-Di Formamide.

Temperature (°C)	Time	cycles
96	10 min	1
96	10 sec	
50	5 sec	25
60	4 min	
4	∞	

Table 2. 11: The thermal cycling profile used for the cycle sequencing of PCR products and plasmids using Applied Biosystems BigDye chemistry.

2.21.3: Sequence assembly and analysis

‘Raw’ sequence data was analysed using the sequencer software, generating SCF files. The SeqMan (DNAstar, Lasergene) sequence analysis package was used to assemble raw SCF files into ‘contigs’ in order to generate a consensus sequence. Alignments of consensus sequences were performed using the Clustal W algorithm in the MegAlign (DNAstar) package. Other sequence manipulations were performed in EditSeq (DNAstar) or BioEdit.

2.22: In vitro transcription (IVT)

2.22.1 IVT of uncapped RNA

Ambion T7 MEGAscript kits were used for the generation of uncapped ssRNA transcripts from plasmids containing the core T7 promoter sequence [5’ – TAATACGACTCACTATA – 3’] (T7p), or PCR products incorporating T7p.

Plasmids used for transcription of RNA, were initially linearised with appropriate restriction enzymes, then used as DNA templates for PCR amplification using primers containing T7p at the 5’ end, followed the target specific sequence. The initiation

efficiency of the T7 polymerase is increased if the first 2-3 nucleotides after the promoter are guanosine bases, where possible these were therefore included as the first nucleotides transcribed.

Large quantities of dsRNA were prepared for generating pools of Dicer siRNA using the Ribomax siRNA generation kit (Promega), again using an RNeasy procedure to remove DNA templates and other transcription reaction components prior to hybridisation of the (+) and (-) strands to form dsRNA.

2.22.2: IVT of capped RNA

The ‘mMESSAGE mACHINE® T7 Ultra’ kit (Ambion) was used to generate 5’ capped RNA transcripts, using 200 ng of gel-purified PCR products containing a 5’ T7 promoter sequence as a template for transcription. After two hours incubation at 37 °C, the DNA template was digested by the addition of 2 units of TURBO DNase to the reaction and incubation for 30. Although this removes the majority of the DNA, the RNA could be further purified by performing a phenol:chloroform extraction.

Where a poly(A) tail was required, tailing reagents supplied with the kit were added to the reactions according to the manufacturer’s instructions, followed by 30 minutes incubation at 37 °C. Transcripts were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the RNA cleanup protocol (section 2.5.4).

2.23: Transfection of Reporter RNAs into BHK-21 cells

BHK-21 cells were seeded in 24 well plates and incubated overnight to reach approximately 90% confluence at the time of infection. Each well was infected by adding 300 µl of 50 % (v/v) virus (original titre approximately 1.0×10^8 PFU/ml, diluted in GMEM minus supplements). The virus was allowed to adsorb for 30 minutes at room temperature, before the inoculum was removed and replaced by 500 µl fresh growth medium. As a negative control mock infections were performed in parallel using unmodified GMEM. At eight hours post-infection, 100 ng of reporter RNA was transfected per well using 0.25 µl Lipofectamine 2000 (Invitrogen) in a total volume of 100 µl OptiMEM (Invitrogen), according to the manufacturer’s conditions. The infected/transfected cells were then incubated for a further 12 hours to allow translation. All treatments were done in triplicate and each experiment was performed at least twice.

2.24: chloramphenicol acetyltransferase (CAT) ELISA

The supernatant was removed from cell sheets, which were then harvested by addition of 300 µl of 1× CAT lysis buffer (Roche), followed by incubation for 20 minutes at room temperature. The lysates were centrifuged at 15,000 × g for 10 minutes at 4 °C to remove cellular debris. 200 µl of the undiluted lysate was then tested in a commercial CAT ELISA kit (Roche) according to the manufacturer's instructions, to determine CAT levels.

2.25: Microscopy

2.25.1: Antibody labelling and coverslip mounting

All incubations were performed at room temperature. Cells were permeabilised for 15 minutes in 0.1 % (v/v) Triton X-100 in PBS, washed once in PBS, and blocked for 0.5 hours in 0.5% (w/v) PBS/BSA blocking buffer. The coverslips were incubated with primary antibodies in blocking buffer, for one hour (at concentrations indicated in Table 2.10), washed three times in PBS, and incubated for one hour with species-specific goat-secondary-antibodies conjugated to Alexa-568 or Alexa-488 (Molecular Probes, Invitrogen Paisley, United Kingdom) diluted 1:200 in blocking buffer. Sub-class specific Alexa-conjugated secondary antibodies, were also used where mouse monoclonal antibodies were used. The coverslips were washed three times in PBS, incubated for 10 minutes in 1 × 4'6'-diamidino-2-phenylindole (DAPI), rinsed in deionised water and mounted onto glass slides using VECTASHIELD®. The coverslips were then sealed onto the slide using clear nail varnish and stored in the dark at 4 °C until examined by microscopy. The antibodies used in this project had all been previously characterised and found to be suitable for use in confocal microscopy. 'No first primary antibody' controls were performed on with/without treatment samples in order to determine the specificity of the antibody. All experiments involving infections included uninfected controls. Experiments incorporating plasmid transfections included 'reagent only' (no plasmid DNA) and 'empty vector' controls.

Antibody	Target	Supplier	Species/Isotype	Working dilution
Orab 1	BTV NS2	N/A (in house)	Rabbit/polyclonal	1:2000
Orab6	BTV cores	N/A (in house)	Guinea pig/polyclonal	1:2000
Orab69	BTV NS1	Polly Roy	Guinea pig/polyclonal	1:1000
α -V5	V5 epitope	Serotec	Mouse mAb/IgG2a	1:1000
GM130	Golgi apparatus	BD Biosciences	Mouse mAb IgG1	1:50
α - α Tubulin	α Tubulin	Sigma	Mouse mAb/IgG1	1:300
α - γ Tubulin ^a	γ Tubulin	Sigma	Mouse mAb/IgG1	1:200
α -Centrin ^a	Centrin	Anke Bruning	Mouse mAb	1:500
α -p150 ^a	p150 ^{glued}	Anke Bruning	Mouse mAb	1:500
α -FOP ^a	FOP	Anke Bruning	Mouse mAb	1:500
α -EB1	EB1	Anke Bruning	Mouse mAb	1:500
Cytochrom C	Cytochrome C	BD Biosciences	Mouse Ab/IgG1	1:500

Table 2. 12: Primary antibodies and their working dilutions.

^aRequires methanol fixation

2.25.2: Confocal microscopy

All confocal microscopy was carried out using a Leica SP2 laser scanning confocal microscope and Leica LCS lite software. Captured images were adjusted in Adobe Photoshop CS2 version 9.0. Live cell imaging was performed using a Leica DMIRE 2.

2.26: Phi6 polymerase reactions

Phi6 polymerase reactions were assembled according to the instructions of the manufacturer (New England Biolabs). Reactions contained 0.2 mM ATP, UTP, CTP, 0.6 mM GTP, 1.5 mM MnCl₂, and phi6 polymerase in the company's reaction buffer (New England Biolabs). *In vitro* transcribed ssRNA template was added to the reactions which were then incubated at 32 °C. The reaction products were analysed using AGE.

Chapter 3: A real-time RT-PCR assay for the pan-detection of BTV

3.1: Introduction

This chapter details the development and evolution of a real-time RT-PCR assay for the pan-detection of bluetongue virus. The results presented form the basis of a published paper (Shaw et al., 2007) and two commercialised RT-PCR kits. The Applied Biosystems BTV RT-PCR detection kit is based upon the published assay (Shaw et al., 2007) whereas the Qiagen *cador* BTV RT-PCR kit, the commercialisation of which is described in this chapter, is based upon a modified version of the published assay.

Many different laboratory-based diagnostic tests can be used to detect if an animal is, or has previously been infected with BTV. These assays can be divided into serology (detection of antibodies to the virus), antigen detection (detection of the virus or viral components), or 'molecular' assays (detection of viral RNA).

BTV virus-species / serogroup specific antibodies are thought to persist for the life of the infected animal and can be detected using enzyme linked immunosorbent assays (ELISA), which are high throughput, quick and relatively easy to perform. The primary group-specific antigen for BTV, which is usually targeted by the antibodies detected in these assays, is the outer core protein (Huismans, 1981; Gumm and Newman 1982; Afshar et al 1992, 1992). VP7 is immunodominant and is relatively conserved, with all serotypes of BTV generating high levels of antibodies to this protein, during infection of their mammalian hosts. Group-specific antibodies to BTV can also be detected by complement fixation, or agar gel immuno-diffusion assays but these are labour intensive and have largely been superseded by more modern techniques.

Antibodies generated against BTV can be further characterised and quantified using virus neutralisation tests. For example a dilution series of serum can be reacted with known amounts of virus (for example 100 TCID₅₀) to test its ability to neutralise the virus. The specificity of these assays is determined by interactions between the neutralising antibodies generated during infection, and BTV outer capsid proteins (particularly VP2). There are 25 distinct serotypes of BTV, which show only low levels of cross-reaction in these assays. In order to confirm the BTV serotype responsible for a previous infection, it is necessary to test the antisera against a panel of BTV 'reference' strains belonging to the different types, which can be a slow and laborious process.

However, serial infections with different BTV serotypes can result in the production of a cross-reactive neutralising response, making it difficult to identify

individual types in areas where multiple viruses are circulating. It is not currently possible to identify and distinguish each of the BTV serotypes by ELISA.

The term 'antigen' (as used in 'antigen' detection assays) can refer to infectious virus or individual protein components of the virus. Viral proteins, including structural components of the virus particle, can be detected using an antigen ELISA (Anderson, 1985; Chand et al., 2009; Stanislawek et al., 1996; Van Wyngaardt and Du Plessis, 1998). Infectious BTV can also be detected isolation in cell cultures, followed by further serological or molecular assays to confirm its identity (Chand et al., 2009).

Molecular assays can be based on the use of labelled oligonucleotide probes that specifically anneal to native and denatured viral RNAs (Brown et al., 1993; Chinsangaram et al., 1992; Dangler et al., 1988; de Mattos et al., 1992; Huismans and Cloete, 1987; Roy et al., 1985; Venter et al., 1995; Venter et al., 1991), or by reverse transcription polymerase chain reactions (RT-PCR) using BTV specific primers (and probes) to amplify and detect viral RNA in a test sample of blood, tissue or cell cultures (Hoffmann et al., 2009a). By targeting primers and/or probes to regions of the genome that are conserved (e.g. Seg-7 - encoding the group specific antigen VP7), or more variable (e.g. Seg-2 - encoding the type specific outer capsid protein VP2), it is possible to use these assays to detect and identify members of the BTV-serogroup, or individual serotypes of BTV (Akita et al., 1992; Anthony et al., 2007; Aradaib et al., 2005; Aradaib et al., 2003; Bandyopadhyay et al., 1998; Jimenez-Clavero et al., 2006; Katz et al., 1993; Mertens et al., 2007; Toussaint et al., 2007a). Although BTV RNA does not persist as long as virus-specific antibodies in the blood of infected animals, the high sensitivity, speed and throughput of RT-PCR assays (particularly 'real-time' assays) makes them particularly useful for rapid detection of the virus and of infected animals.

Although competition ELISA (cELISA) can be used to detect BTV specific antibodies in serum samples (Anderson, 1984; Anderson et al., 1993), these antibodies are not generated until 7-10 days post infection. Serological methods to detect and 'type' virus in blood can take several weeks, involving virus isolation (e.g. in embryonated chicken eggs, or on KC cells), adaptation to mammalian cell culture and virus or serum neutralisation tests (VNT or SNT). These assays depend on the availability of highly characterised antigens and antibodies for the cELISA (to identify the virus species / serogroup), and reference antisera or reference strains for all 24/25 distinct BTV serotypes for neutralisation assays (to identify serotype).

Assays based on RT-PCR, can detect BTV RNA in clinical samples (e.g. blood or spleen) without virus isolation and do not require standardised serological reagents, making them more accessible to different laboratories, and potentially less-expensive overall. However, many published RT-PCR based methods have not been fully evaluated for the detection of different BTV serotypes or topotypes and some published methods will only detect certain BTV strains (Aradaib et al., 2005; Aradaib et al., 1998; Aradaib et al., 2003; Billinis et al., 2001; Dangler et al., 1990; Jimenez-Clavero et al., 2006; Orru et al., 2004; Shad et al., 1997; Wade-Evans et al., 1990; Zientara et al., 2002). The majority of published RT-PCR primer sets target BTV Seg-5 (Seg-5 - coding for NS1), or genome segment 7 (Seg-7 - coding for VP7) (Anthony et al., 2007; Aradaib et al., 2005; Aradaib et al., 2003; Jimenez-Clavero et al., 2006). Although these genome segments are relatively conserved across the BTV virus-species (Maan et al 2009- in preparation), they are sufficiently divergent between distinct orbiviruses to remain BTV specific. VP1, VP3, VP4, NS1 and NS2 are also highly conserved (Huismans and Cloete, 1987) and can be used as target for RT-PCR based 'group' assays. Seg-5 is also recommended as an RT-PCR target by the Office International des Epizooties (OIE), (OIE, 2004)).

Real-time RT-PCR (rRT-PCR) assays offer certain advantages over traditional gel-based RT-PCR methods. The target amplicon is usually smaller, reducing the potential for target degradation. Detection of a specific gene sequence by rRT-PCR involves monitoring fluorescence generated by cleavage of a target specific oligonucleotide probe during amplification. This format eliminates the need to open the reaction tube post-amplification, for either a nested step or final gel analysis of the cDNA products, greatly reducing the risk of cross-contamination. The rRT-PCR format is therefore particularly well suited for diagnostic procedures where high reliability, throughput, sensitivity and specificity are required. However, real-time PCR probes can be very sensitive to probe-target mismatches and even relatively few base differences can potentially give false negative results (Jimenez-Clavero et al., 2006). This suggests that the more highly conserved regions of the conserved BTV genome segments are likely to provide the most appropriate targets for development of BTV specific rRT-PCR assays. BTV Seg-1 encodes the viral polymerase, one of the most highly conserved proteins of all members of the family *Reoviridae* (Huang et al., 1995). However, Seg-1 also contains regions of sequence that remain BTV-specific.

This chapter presents data pertaining to the development, evaluation and commercialisation of a real-time RT-PCR assay for the detection of BTV genome segment 1 (Seg-1).

3.2: Sequence analysis of Segment-1 of BTV and EHDV

At the inception of this project only seven Seg-1 sequences were available through GenBank (Figure 3.3). Comparisons of these data identified a region of the segment that was highly conserved across all of the available sequences. This region, spanning nt 291-387 (numbered according to GenBank accession number AY154458), satisfied requirements for a rRT-PCR probe (e.g. high melting temperature) and there were likely flanking primer sites, which would generate a small cDNA amplicon. Although promising, more sequence data, including sequences for closely related non-BTV viruses, were required to confirm that this site would be both broadly reactive within the BTV serogroup, but would not react with other orbiviruses thus providing specificity for BTV as a species.

RT-PCR primers ORBI-UNI-F and ORBI-UNI-R (Table 1) were designed flanking either side of the target region of Seg-1, that were predicted to amplify a 412bp cDNA amplicons from BTV, African horsesickness virus (AHSV) and epizootic haemorrhagic disease virus (EHDV) (Mertens, 2005).

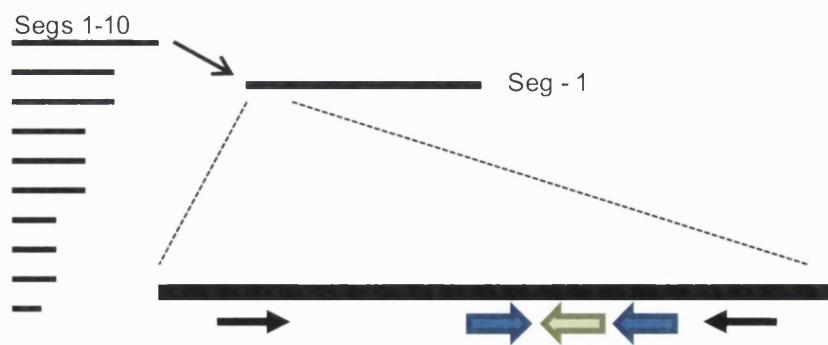


Figure 3. 1: A Cartoon depicting the location of the real-time RT-PCR assay located in Seg-1.

RT-PCR primers ORBI-UNI-F and ORBI-UNI-R (black arrows) were used to produce an amplicon encompassing the real-time RT-PCR primers (blue arrows) and probes (green arrow). The amplicons generated for a selection of viruses were sequenced to determine the suitability of the real-time primers and probes.

Twenty six different BTV isolates and two EHDV isolates were selected from the orbivirus reference collection at IAH Pirbright, based on their geographical origins (topotype) (see: http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/orbiviruses.htm) including at least one representative from each BTV serotype. The 412bp amplicons were generated for each of the selected viruses and sequenced using the same PCR primers (ORBI-UNI-F and ORBI-UNI-R, Table 1) and Beckman CEQ8000 chemistry (see Figure 3.1, and methods section 2.21.1a). The resulting sequences were assembled using SeqMan (DNAstar, Lasergene) then further aligned and analysed using Bioedit.

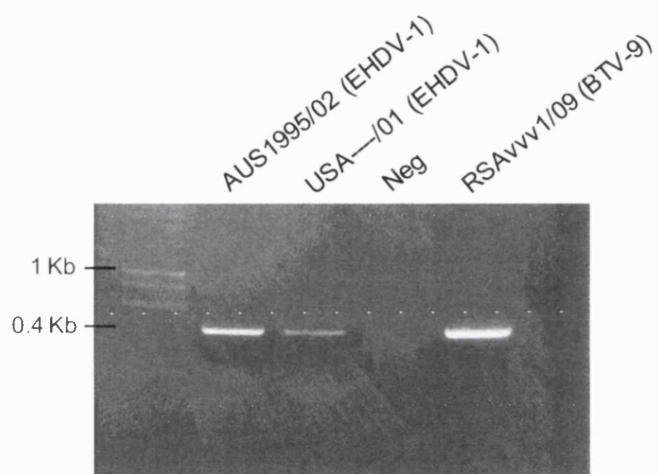


Figure 3. 2: 412bp cDNA products from BTV and EHDV genome segment 1.
Amplification of a 412 bp fragment of Seg-1, encompassing the selected target region for a rRT-PCR amplicon. Two isolates of EHDV and one isolate of BTV were amplified using primer pair ORBI-UNI-F and ORBI-UNI-R. The RT-PCR products were analysed and purified by 1% agarose gel electrophoresis (1% AGE) then re-extracted from the gel before being sequenced using the same primers.

Purpose		Oligo name	Sequence (5' – 3')	Genomic Location ^a
BTV SEG1 RSA (east)	Forward Primer	BTVrsa 291-311F	GCGTTCGAAGTTACATCAAT	291-311
	Reverse Primer	BTVrsa 387-357R	CAGTCATCTCTCTAGACACTCTATA ATTACG	387-357
BTV SEG1 UNI (west)	Forward Primer	BTVuni 291-311F	GCTTTGAGGTGTACGTGAAC	291-311
	Reverse Primer	BTVuni 381-357R	TCTCCCTTGAAACTCTATAATTACG	381-357
BTV SEG1 PROBES	Probe RSA	RSA-BTV 341-320	CGGATCAAGTTCACTCCACGGT	341-320
	Probe 323	BTV 346-323	TCCTCCGGATCAAGTTCACTCCAC	346-323
RT-PCR + sequencing	Forward Primer	ORBI-UNI-F	YMATCACCGTGCAAGGT	19-35
	Reverse Primer	ORBI-UNI-R	TGCATYTCGTTTTMGC	430-414

Table 3. 1: Primer and probe sequences used for RT-PCR, sequencing and real-time RT-PCR assays for detection of BTV.

The primers ORBI-UNI-F and ORBI-UNI-R were used for RT-PCR amplification and sequencing of the 412bp cDNA amplicons from BTV Seg-1. This region was selected as a target for design of real-time RT-PCR primers and probes. Combining the RSA (east) and UNI (west) sets of primers, with both probes, in a single assay allowed detection of all of the BTV strains tested regardless of serotype or topotype. Separate reactions using only primer set ‘RSA’ or primer set ‘UNI’ with both probes, allowed eastern or western BTV strains to be identified and distinguished (respectively).

^a Genome location according to GenBank accession number AY154458.

The most immediately conspicuous feature arising from the sequence data was that the consensus sequences for this region were divided into two distinct groups, corresponding to BTV strains from eastern and western origins (with 78.3% to 87.6% identity between groups). The virus isolates in the eastern group (from the Middle-east, Asia and Australasia, Figure 3.3(B)) showed significantly higher identity (87.6-96.9%) to the published sequence for Seg-1 from a Taiwanese isolate (**AY493686**, unknown serotype). Isolates in the western group of BTV isolates (originating from Africa and the Americas - BTV-11 (**L20445**), BTV-13 (**L20446**), BTV-17 (**L20447**), BTV-2 (**L20508**), BTV-10 (**NC_006023** and **X12819**)) showed a high level of similarity to the Seg-1 sequence of BTV-2 from Corsica (92.8-97.9% identity relative to **AY154458**, Figure 3.3(A)).

	BTVuni 291-311F	Probe RSA	Probe 323	BTVuni 381-357R
A)		CACCTCACTTGAACCTAGGCTCCT TGCCACCTCACTTGAACCTAGGC		GCATTAATAATCTCAAGATTCCCTCT
BTV-2 (from AY154458)	G.....A.....		
BTV-2 (from L20508)	G.....C.....	
BTV-3 [NIG1982/06 (EF059721)]	G..A.....C..C.....	
BTV-4 [ARG2002/01 (EF059722)]A.....	G.....A.....C.....	
BTV-4 [SPA2005/05 (EF059723)]	G.....C.....	
BTV-5 [NIG1982/04 (EF059724)]	G.....C..C.....	
BTV-6 [RSArrrr/06 (EF059725)]	G.....C.....	
BTV-7 [RSArarr/07 (EF059726)]	G.....		
BTV-8 [RSArarr/08 (EF059727)]G.....A.....		
BTV-8 [NET 2006/04	G.....C.....	
BTV-9 [RSAvvv1/09 (EF059728)]A.....	G.....A.....C..C.....	
BTV-9 [RSAvvv2/09]A.....	G..A.....C..C.....	
BTV-10 (from NC_006023)	G.....		
BTV-10 (from X12819)	G.....		
BTV-11 (from L20445)	G.....		
BTV-12 [ZIM2003/04 (EF059730)]	G.....C.....G.....	
BTV-12 (NIG1982/09)A.....	G..A.....C.....C..C..C.....	
BTV-13 (from L20446)	G.....		
BTV-14 [CAR1982/04 (EF059731)]	G.....C..C.....	
BTV-15 [ZIM2003/08 (EF059732)]	G.....G.....A.....	
BTV-17 (from L20447)	G.....		
BTV-18 [RSArarr/18 (EF059735)]	G.....		
BTV-19 [RSArarr/19 (EF059736)]C..C..C.....	
BTV-22 [NIG1982/11 (EF059739)]T	G.....C.....C..C.....	
BTV-24 [RSArarr/24 (EF059741)]	G.....C.....	
BTV-16 [RSAvvvv/16 (EF059734)]G..C..A..T..A..C..T	GAA..TAGAG..CT..A..G..A..A..G..T..A..	
AHSV-9 (from NC_006021)AG..A..TA..T..T..T..GA	GAA..TAGAG..CT..A..G..A..A..CGC..ATGAT..GTG	
AHSV-9 (from U94887)AG..A..TA..T..T..T..GA	GAA..TAGAG..CT..A..G..A..A..CGC..ATGAT..GTG	
EHDV-1 [AUS1995/02 (EF059742)]AC..A.....TA.....	G..GTCA..GAG..GT..G..G.....A..C..AG..ACGG..C..AC	
EHDV-1 [USA---/01 (EF059743)]TG..C..A..T..T..T..GA	..G..GCAAGAA..CT..A..A..A..GATG..T..AC	
IBARAKI (from AB186040)T..AC..A..T..T..T..GA	G..GCCA..GAA..T..GA.....A..AG..GCA..G..C..AC	
	BTVuni 291-311F			BTVuni 387-357R
B)		CGGTTCGAAGTTTACATCAAT		GCATTAATAATCTCACAGATCTCTACTGAC
BTV-1 [GRE2001/02 (EF059720)]T.....A.....A.....		
BTV TAIWAN from AY493686)G..C.....			
BTV-9 [KOS2001/04 (EF059729)]			
BTV-16 [RSArarr/16 (EF059733)]			
BTV-16 [RSAvvvv/16 (EF059734)]			
BTV-20 [RSArarr/20 (EF059737)]C.....A..C..C..G.....A.....	
BTV-21 [RSArarr/21 (EF059738)]TC.....A..C..G.....A.....	
BTV-23 [IND1988/03 (EF059740)]C.....			
BTV-7 [RSArarr/07 (EF059726)]T..T..G..G..G..G..C	G.....T..A..G.....	
AHSV-9 (from NC_006021)AG..A..TA.....TG..T..GA	GAA..TAGAG..CT..A..G..A..A..CGC..T..A..GAT..GTG..CACC	
AHSV-9 (from U94887)AG..A..TA.....TG..T..GA	GAA..TAGAG..CT..A..G..A..A..CGC..T..A..GAT..GTG..CACC	
EHDV-1 [AUS1995/02 (EF059742)]T..AC..G..T..G..C	G..GTCA..GAG..GT..G..G.....A..C..AG..ACGG..C..ACATG..G	
EHDV-1 [USA---/01 (EF059743)]T..C..A..T..G..C	..G..GCAAGAA..CT..A..A..A..A..CA..G..T..ACACG..	
IBARAKI (from AB186040)T..AC..G..A..T..G..C	G..GCCA..GAA..T..GA.....A..AG..GAG..C..ACATG..	
	BTVuni 387-357R			

Figure 3. 3: Sequence alignment of the real-time RT-PCR primer and probe footprints for genome segment 1 of different orbiviruses.

Sequences were aligned for of the targeted region of Seg-1 from BTV (types 1-24), EHDV (eastern and western strains of type 1 and Ibaraki virus type 2) and AHSV (type 9).

Section A) represents viruses of western origin aligned against primer set UNI, but includes BTV-16 (RSAvvvv/16) as a representative eastern virus. **Section B)** represents viruses of eastern origin aligned against primer set RSA, but includes BTV-7 (RSArarr/07) as a representative western virus. Probe footprints for A) and B) are both aligned against probes RSA and 323.

3.3: Development of a BTV specific real time RT-PCR assay

3.3.1: Primers/probes design

Potential target footprints for development of RT-PCR primers (nt 291-311 and nt 381-357 - Table 3.1) were identified within the conserved region of Seg-1. Both primer sites have two consensus sequences (representing either eastern or western BTV topotypes respectively) and two distinct sets of primers were designed to amplify

sequences from these different groups (Table 3.1). A highly conserved sequence (nt 341-323) was also selected as the target for a TaqMan® probe (which is identified as RSA-BTV 341-320 in Table 3.1). Sequence comparisons indicated that these target sequences for primers and probes are significantly different from those of the same regions of closely related *Orbivirus* species AHSV and EHDV (Figure 3.3). The expected products that would be amplified using these primer sets are 97bp (eastern PCR) or 91bp (western PCR) respectively. Using these primers, ‘eastern-specific’ and ‘western-specific’ assays can be run separately but in parallel, or combined into a single reaction, to allow the detection of any BTV strains from either group (Figure 3.4).

Initially experiments used only the RSA probe in the mastermix reducing the chance of error or interference/competition between oligos. The RSA probe matched the eastern strains, (which includes RSAvvvv/16, the virus used for the majority of this PhD) and therefore the probe contains a 3’ nucleotide mismatch with all of the western strains with the exception of RSArrrr/08 and RSArrrr/19 (Figure 3.3).

3.3.2: Optimisation of the Seg-1 BTV group-specific real-time RT-PCR assay

PCR assays using the eastern (RSA) and western (UNI) genotype-specific primer sets (Table 3.1) were optimised separately. The optimal concentration of each primer was determined by performing chequerboard experiments where every combination of each concentration was tested, with the result that 20 pmols of each primer was optimal. The two sets of primers were subsequently included in a single combined reaction, to allow the detection of either eastern or western viruses in a single tube reaction. The impact of altering the magnesium sulphate ($MgSO_4$) concentration was also assessed. It was found that adjusting the $MgSO_4$ concentration to 4 mM improved the efficiency of the reaction. The addition of ROX as a reference dye was found not to impact upon the reaction efficiency. The Superscript III/Platinum *Taq* one-step RT-PCR kit (Invitrogen) was used for all rRT-PCR assays. Each 19 μ l of reaction mix contained the following: 12.5 μ l of 2x reaction buffer mix (kit), 20 pmols of each primer, 2.5 pmols each probe, 0.5 μ l Mg_2SO_4 (kit), 0.5 μ l diluted ROX reference dye (kit) and 0.5 μ l of the Superscript III/Platinum *Taq* enzyme mix (kit).

When only single probe and primer sets were included, the remaining volume was substituted with nuclease free water. Mastermix was distributed to the wells of Stratagene Mx3005 reaction plates. Mastermix preparation and distribution into wells was performed in a dedicated PCR hood in a different laboratory from the RT-PCR

assembly hood. The 6 μ l of denatured RNA was added to the reaction mix and the reaction capped using optical caps (Stratagene). Amplification was carried out in an Mx3005P PCR machine (Stratagene) using the following optimised programme: 55 °C for 30 min, 1 cycle (reverse transcription), 95 °C for 10 min, 1 cycle (denaturation of the Superscript III and activation of the Platinum *Taq* DNA polymerase) and 50 cycles of 95 °C for 15 s and 60 °C for 1 minute. Fluorescence was measured at the end of the 60 °C annealing/extension step. Cycle threshold (Ct) values for each sample were determined from the point at which fluorescence breached a threshold fluorescence line. Negative results are described as 'No Ct'.

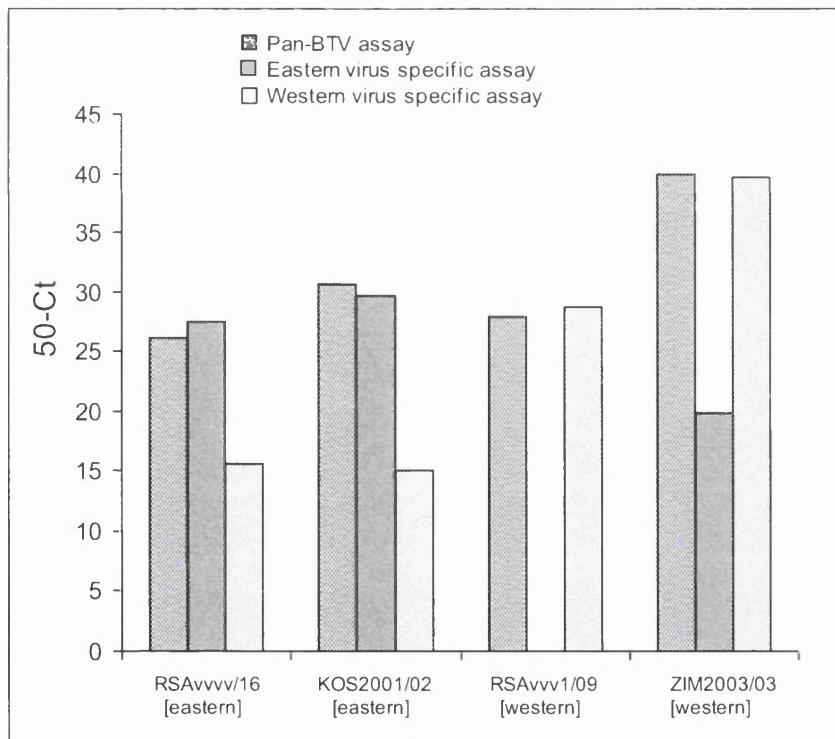


Figure 3. 4: Evaluation of single and duplex real time RT-PCR assays targeting Seg-1, for detection of two eastern and two western viruses.

By including only one Seg-1 primer set (RSA or UNI) it is possible to distinguish viruses of eastern origin from those of western origin. The appropriate primer set gives Ct values similar to those of the pan-BTV assay. The data shown includes two serotype 9 viruses (KOS2001/02 [East] and RSAvvv1/09 [west]) which can be distinguished using this approach. Although a 'No Ct' value was obtained for RSAvvv1/09 using the eastern (RSA) primer set, the remaining heterotypic primer sets gave Ct values, (albeit higher/weaker) for every virus tested.

3.4: Evaluation

3.4.1: Analytical sensitivity

The Analytical sensitivity of the rRT-PCR assay targeting BTV Seg-1 (i.e. the number of ssRNA molecules that can be detected) was assessed using *in vitro* transcribed BTV RNA as a target. A 412 bp fragment (nt 19-430) of Seg-1 from RSAvvvv/16 was first amplified using primers ORBI UNI F and ORBI UNI R (Table 3.1). The resulting RT-PCR product was then cloned into pGEM T-easy (Promega) and sequenced in both directions using M13 primer sites available on the plasmid. A clone with a sequence demonstrating 100 % identity with the real-time primer and probe footprints was selected and digested using SalI. The linearised plasmid was subsequently used as a template for *in vitro* transcription (MegaScript, Ambion). Following DNase digestion to remove the plasmid template, the RNA was purified using an RNeasy spin column (Qiagen). An aliquot of the purified transcripts was assessed and quantified using the Agilent Bioanalyser. The resulting quantification was used to calculate the copy number. To determine the minimum number of RNA copies detectable, a series of rRT-PCR reactions was assembled containing between 9×10^0 and 9×10^{11} copies of input ssRNA. The results demonstrated a good linear relationship between RNA copies and Ct value indicating that the RT-PCR reaction was efficient. It was found that the assay would easily detect 100 copies and also down to approximately 10 copies. Mertens *et al* (1996) calculated that a cell culture ‘infectious unit’ of disaggregated BTV for BHK-21 cells comprised 1000 BTV particles. As each virus particle contains two copies of ssRNA to make the dsRNA genome, one infectious unit of BTV equates to 2000 copies of RNA. If the minimum number of copies required to obtain a positive result is ten, it can theoretically be calculated that this sensitivity would equate to five virus particles. However, as the assay will detect the ssRNA that is also synthesised during infection, this level of detection would be sufficient to detect a single replicating virus. Overall, these data imply that the rRT-PCR represents an assay with much greater sensitivity than virus isolation in cell culture.

3.4.2: Diagnostic sensitivity

‘Diagnostic sensitivity’ is defined as the ability of an assay to detect all strains of a specific pathogen. Assays with high diagnostic sensitivity are therefore capable of detecting a diverse range of different samples / isolates / serotypes / topotypes of the targeted pathogen.

A selection of geographically distinct BTV isolates, including isolates of all 24 established BTV serotypes and different topotypes (as indicated in Table 3.2) was therefore used to test the diagnostic sensitivity of the dual probe rRT-PCR assay. The RNA of these viruses was already available as they had been isolated for use in a previous study using conventional RT-PCR procedures targeting Seg-7 (Anthony et al., 2007). Two sets of data were generated, using either the RSA probe, or both the RSA and UNI probes, with combined eastern and western primers sets. The results demonstrated that the RSA probe alone was capable of detecting representatives of the 24 established BTV serotypes, including both eastern and western strains (with 94.6 % of virus isolates testing positive).

Although a 'No Ct' result was initially obtained using the dual probe rRT-PCR assay with RNA from three isolates (RSAvvv1/09 [BTV-9], ITL2003/01 [BTV-9] and NIG1982/09 [BTV-12]), electrophoretic analysis of the reaction products showed successful amplification of the target region of Seg-1 from each of these samples, suggesting that the probe had not bound to its target region in Seg-1 of these viruses. Subsequent sequencing of the amplicons generated using primers ORBI UNI F and ORBI UNI R identified mismatches between the probe and its target footprint for all three isolates (Figure 3.3). A second probe (BTV 346-323 – moved 3 bases down-stream and extended by a further two bases at its 3' end – Figure 3.3), was designed to match the same region of Seg-1 but improve detection of these three isolates. When this second probe was tested in a combined assay (along with the original probe and both primer sets) it gave reliable detection of RSAvvv1/09 and NIG1982/09 with no reduction in assay performance (Table 3.2). In each case, diluting the RNA sample improved the detection of the virus, thus implying that either the RNA was too concentrated or that the sample contained a RT-PCR inhibitor. The reaction mixture containing both probes detected all of the viruses (including ITL2003/01) tested thus demonstrating 100% diagnostic sensitivity.

The dual probe assay was also tested for its ability to detect BTV RNA in homogenised *Culicoides*. RNA was extracted using the QIAamp viral RNA kit from a selection of infected *Culicoides* homogenates prepared as part of a separate study (Carpenter et al., 2006). The dual probe RT-PCR assay was found to be capable of detecting the BTV RNA in the homogenates (Table 2).

IAH-Pirbright Reference collection number	Origin/Source	Serotype ^a	Mixed Probes (BTV 346-323 & RSA 341-320P)	Probe BTV- RSA 341-320P
RSArrr/01	S. Africa	BTV-1	23.05	13.70
GRE2001/01	Lesbos	BTV-1	17.23	20.15
GRE2001/02	Lesbos	BTV-1	18.39	20.42
IND1992/02	India	BTV-1	18.21	21.60
IND2001/01	Chennai	BTV-1	22.27	23.80
SUD1987/01	Sudan	BTV-1	14.34	15.08
NIG1982/01	Nigeria	BTV-1	13.19	14.57
AUS1981/01	Australia	BTV-1	16.04	17.61
GRE2001/03	Greece	BTV-1	19.21	
GRE2001/04	Greece	BTV-1	16.31	17.10
Algeria 1 (spleen)	Algeria	BTV-1	18.23	
RSArrr/02	S. Africa	BTV-2	17.87	16.14
ZIM2003/01	Zimbabwe	BTV-2	9.75	9.69
ITL2002/07	Sicily	BTV-2	10.06	12.05
ITL2002/06	Raccuja	BTV-2	14.36	
ITL2002/05	S. Angelo di Brolo	BTV-2	9.73	9.39
ITL2002/04	S. Pietro Patti	BTV-2	7.87	
ITL2002/03	Novara di Sicila	BTV-2	13.09	
ITL2002/02	Novara di Sicila	BTV-2	12.94	
ITL2002/01	Novara di Sicila	BTV-2	17.67	
IND1982/01	India	BTV-2	16.11	
NIG1982/02	Nigeria	BTV-2	19.5	
FRA2001/03	Corsica	BTV-2	23.88	
FRA2001/06	Corsica	BTV-2	11.92	11.05
TUN2000/01	Tunisia	BTV-2	16.31	21.18
SAD2001/02	Sardinia	BTV-2	18.61	16.94
SAD2002/01	Sardinia	BTV-2	21.46	
SAD2001/04	Sardinia	BTV-2	16.58	
SAD2002/03	Sardinia	BTV-2	17.8	
SAD2001/06	Sardinia	BTV-2	20.21	
RSArrr/03	S. Africa	BTV-3	16.04	13.78
ZIM2002/01	Zimbabwe	BTV-3	18.37	18.12
ZIM2002/02	Zimbabwe	BTV-3	21.65	22.63
NIG1982/06 ^b	Nigeria	BTV-3	18.39	20.71
RSArrr/04	S. Africa	BTV-4	17.62	15.28
RSAvvv3/04	S. Africa	BTV-4	21.53	22.44
RSAvvv5/04	S. Africa	BTV-4	23.91	
RSAvvv7/04	S. Africa	BTV-4	20.52	21.63
TURvvv/04	Turkey	BTV-4	11.42	11.33
GRE1999/02	Lesbos	BTV-4	15.96	
GRE1999/03	Lesbos	BTV-4	15.6	13.81
GRE1999/05	Pieria	BTV-4	16.5	
TUR1978/01	Turkey	BTV-4	15.03	15.10
SUD1983/01	Sudan	BTV-4	17.46	
GRE2000/03	Greece	BTV-4	20.48	
GRE2000/06	Greece	BTV-4	23.55	
ARG2002/01 ^b	Argentina	BTV-4	14.33	14.77
ARG2002/02	Argentina	BTV-4	15.79	
ARG2002/03	Argentina	BTV-4	16.54	17.11
ARG2002/04	Argentina	BTV-4	18.22	
SPAIN 1 (blood) ^d	Spain	BTV-2	28.41	
SPAIN 2 (blood) ^e	Spain	BTV-4	24.21	
SPAIN 3 (blood) ^f	Spain	BTV-4	25.98	22.65
SPAIN 4 (blood) ^g	Spain	BTV-4	26.39	23.44
SPAIN 5 (blood)	Spain	BTV-4	25.02	22.47
SPAIN 6 (blood)	Spain	Isolation -ve at CRL	27.16	23.32
GRE1999/10	Greece	BTV-4	20.75	
GRE1999/11	Greece	BTV-4	19.07	
RSArrr/05	S. Africa	BTV-5	18.12	14.61
NIG1982/03 ^b	Nigeria	BTV-5	17.92	
NIG1982/04 ^b	Nigeria	BTV-5	16.71	18.89
CAR1982/02	Cameroon	BTV-5	18.49	20.51
RSArrr/06 ^b	S. Africa	BTV-6	16.29	14.25
RSArrr/07 ^b	S. Africa	BTV-7	18.49	19.25
RSArrr/08 ^b	S. Africa	BTV-8	20.75	16.63

NIG1982/07	Nigeria	BTv-8	16.16	
KEN---/01	Kenya	BTv-8	17.55	NO Ct
NET2006/01	Netherlands	BTv-8	20.35	
RSArrr/09	S. Africa	BTv-9	16.65	15.90
RSAvvv1/09 ^b	S. Africa	BTv-9	25.25	NO Ct
RSAvvv2/09 ⁱ	S. Africa	BTv-9	24.00 [NO Ct]	
RSAvvv9/09	S. Africa	BTv-9	18.78	
ITL2003/01 ⁱ	Sicily	BTv-9	20.88 [NO Ct]	NO Ct
GRE1999/06	Halkidiki	BTv-9	9.82	7.07
TUR2000/11	Turkey	BTv-9	8.37	
TUR2000/04	Turkey	BTv-9	20.17	
TUR2000/05	Manisa	BTv-9	21.23	
TUR2000/03	Manisa	BTv-9	21.34	
BUL1999/01	Bulgaria	BTv-9	18.14	18.34
GRE2000/02	Greece	BTv-9	6.54	
BOS2002/01	Vlasenica	BTv-9	19.03	18.52
BOS2002/02	Vlasenica	BTv-9	18.7	
BOS2002/03	Vlasenica	BTv-9	16.42	
BOS2002/04	Vlasenica	BTv-9	17.39	
KOS2001/01	Kosovo	BTv-9	16.85	
KOS2001/02	Kosovo	BTv-9	18.18	
KOS2001/03	Kosovo	BTv-9	17.4	
KOS2001/04 ^b	Kosovo	BTv-9	16.31	
KOS2001/04 ^c	Kosovo	BTv-9	19.3	20.57
KOS2001/04 ^c	Kosovo	BTv-9	22.7	22.33
KOS2001/04 ^c	Kosovo	BTv-9	25.07	26.18
KOS2001/04 ^c	Kosovo	BTv-9	26.36	27.03
RSArrr/10	S. Africa	BTv-10	15.52	15.62
RSArrr/11	S. Africa	BTv-11	16.25	15.06
ZIM2003/05	Zimbabwe	BTv-11	9.04	11.70
NIG1982/08	Nigeria	BTv-11	16.29	16.67
ZIM2003/02	Zimbabwe	BTv-11	11.21	
ZIM2003/03	Zimbabwe	BTv-11	10.25	
RSArr/12	S. Africa	BTv-12	16.93	14.50
ZIM2003/04 ^b	Zimbabwe	BTv-12	10.34	
NIG1982/09	Nigeria	BTv-12	18.18	NO Ct
KEN----/-	Kenya	BTv-12	18.65	
RSArrr/13	S. Africa	BTv-13	17.08	17.44
RSArrr/14	S. Africa	BTv-14	15.34	15.02
CAR1982/04 ^b	Cameroon	BTv-14	15.91	16.78
RSArrr/15	S. Africa	BTv-15	17.21	15.76
ZIM2003/08 ^b	Zimbabwe	BTv-15	9.64	6.93
ZIM2003/09	Zimbabwe	BTv-15	8.00	
RSArrr/16 ^b	S. Africa	BTv-16	27.19	
TUR2000/09	Izmir	BTv-16	9.77	
TUR2000/08	Manisa	BTv-16	9.26	
TUR2000/01	Izmir	BTv-16	19.1	20.58
TUR2000/02	Manisa	BTv-16	19.15	
TUR2000/10	Izmir	BTv-16	20.19	
NIG1982/10	Nigeria	BTv-16	18.55	20.10
RSAvvv/16 ^b	S. Africa	BTv-16	23.88	22.07
CYP2004/001	Cyprus	BTv-16	15.82	18.19
RSArrr/17	S. Africa	BTv-17	14.31	13.17
RSArrr/18 ^b	S. Africa	BTv-18	15.34	16.71
RSArrr/19 ^b	S. Africa	BTv-19	17.74	16.03
RSArrr/20 ^b	S. Africa	BTv-20	18.17	16.53
AUS1978/01	Australia	BTv-20	21.45	21.21
RSArrr/21 ^b	S. Africa	BTv-21	22.03	21.15
RSArrr/22 ^b	S. Africa	BTv-22	18.24	17.27
NIG1982/11 ^b	Nigeria	BTv-22	18.14	19.80
RSArrr/23	S. Africa	BTv-23	16.5	14.37
IND1998/01	Bangalore	BTv-23	15.76	
IND1988/02	Rahuri	BTv-23	16.46	19.27
IND1997/01	Bangalore	BTv-23	16.08	
IND1988/03 ^b	Rishikesh	BTv-23	13.19	
RSArrr/24 ^b	S. Africa	BTv-24	15.4	14.13

Table 3. 2: Testing samples of different Bluetongue viruses by Seg-1 real time RT-PCR.

Ct values obtained using either probe RSA-BTV 341-320 by itself, or combined with RSA 341-320P in a single reaction. In every case both primer sets RSA and UNI were included in the reaction. Viruses sequenced as part of the study are indicated.

^a BTV = Bluetongue virus;

^b Isolates sequenced as part of this study.

^c RNA isolated from individually homogenised *Culicoides* midges infected with BTV-9.

^{d-h}BTV was isolated from Spanish blood samples 1-5 resulting in virus isolates SPA2005/01 to SPA2005/05 respectively.

ⁱ The sample preparations for these samples required dilution to be efficiently detected.

More information about individual virus samples (catalogued by their IAH-Pirbright Reference collection number) is available at: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/orbiviruses.htm

^j The Ct given is for RNA isolated from homogenised spleen material from which ALG2006/01 was subsequently isolated.

3.4.3: Assay Specificity

An assay's 'specificity' refers to its ability to discriminate between BTV and other RNAs that may be present in a test sample. It is important that assays are highly 'specific' in order to provide accurate diagnosis and avoid false positive results. Although *in silico* analysis of sequence data suggested that neither the primers nor probes targeting BTV-Seg-1 had sufficient homology to bind RNA from other orbivirus species, it is still necessary to confirm this experimentally. It is also important that the assay should not give false positive results with host cell sequences.

EHDV also infects ruminants, is the most closely related orbivirus to BTV and therefore considered to be the most likely source of cross-reactions in diagnostic samples. Eastern and western strains of EHDV, as well as different serotypes of AHSV, isolates of five other *Orbivirus* species and five unassigned orbiviruses were all tested using the combined assay (with both primer sets and single or combined probes). The assay was also tested with nucleic acid samples extracted from EDTA treated blood from uninfected sheep or cattle.

IAH-Pirbright Reference collection number	Origin / Source	Serotype ^a	Sample material ^b	Mixed Probes (BTV 346-323 & RSA 341-320P)	Probe BTV-RSA 341-320P
RSArrah/01	S. Africa	AHSV-1	BHK	NO Ct	NO Ct
RSArrah/02	S. Africa	AHSV-2	BHK	NO Ct	NO Ct
RSArrah/03	S. Africa	AHSV-3	BHK	NO Ct	NO Ct
RSArrah/04	S. Africa	AHSV-4	BHK	NO Ct	NO Ct
RSArrah/05	S. Africa	AHSV-5	BHK	NO Ct	NO Ct
RSArrah/06	S. Africa	AHSV-6	BHK	NO Ct	NO Ct
KENrrah/07	S. Africa	AHSV-7	BHK	NO Ct	
PAKrrah/09	S. Africa	AHSV-9	BHK	NO Ct	NO Ct
Bryanstone	-	EEV-1	BHK	NO Ct	NO Ct
Cascara	-	EEV-2	BHK	NO Ct	NO Ct
Kaaplas	-	EEV-4	BHK	NO Ct	NO Ct
Kyalami	-	EEV-5	BHK	NO Ct	NO Ct
Potchefstroom	-	EEV-6	BHK	NO Ct	NO Ct
AUS1995/02 ^c	-	EHDV-1	BHK	NO Ct	NO Ct
USA----/01 ^c	-	EHDV-1	BHK	NO Ct	NO Ct
Andasibe virus	-	Orbivirus	unknown	NO Ct	NO Ct
Chobar Gorge virus	-	Orbivirus	unknown	NO Ct	NO Ct
Japanaut virus	-	Orbivirus	unknown	NO Ct	NO Ct
Matucare virus	-	Orbivirus	unknown	NO Ct	NO Ct
Tembe virus	-	Orbivirus	unknown	NO Ct	NO Ct
Tracambe virus	-	Orbivirus	unknown	NO Ct	NO Ct
Wad Medani virus	-	Orbivirus	unknown	NO Ct	NO Ct
Uninfected ovine	-	-	Blood	NO Ct	NO Ct
Uninfected bovine	-	-	Blood	NO Ct	NO Ct
Positive control	S. Africa	BTV ^d	BHK	10.54	11.65

Table 3. 3: The specificity of BTV Seg-1 RT-PCR.

A variety of non-BTV orbiviruses were tested in order to confirm specificity for BTV nucleic acid. Ct values obtained using either probe RSA-BTV 341-320 alone or both probes incorporated into a single reaction. In every case both primer sets RSA and UNI were included in the reaction. Viruses sequenced as part of the study are indicated.

^a AHSV = African Horse Sickness Virus; BTV = Bluetongue Virus; EEV = Equine Encephalosis virus; EHDV = Epizootic Haemorrhagic Disease virus.

^b BHK = BHK-21 cell culture supernatant; Blood = EDTA treated whole blood.

^c Isolates sequenced as part of this study.

^d The positive controls were BTV isolates TUR2000/09 (BTV-16) and ZIM2003/04 (BTV-12) for assays using the mixed probes or the RSA probe alone respectively.

A 'No Ct' value was obtained when any non-BTV nucleic acid was tested using the optimised assays including both probes or probe BTV-RSA 341-320P alone (Table 3.3). If the probes bind with insufficient affinity it is potentially possible to obtain a No Ct value even when amplification takes. To determine that there was no amplification of non-BTV sequences, the post-rRT-PCR samples were run on a 3% agarose gel. No bands were evident except for samples containing BTV (Figure 3.5).

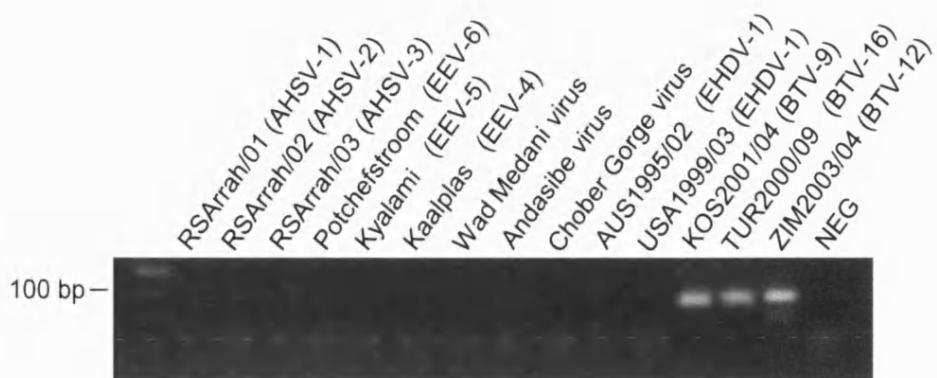


Figure 3. 5: AGE of cDNAs from the Seg-1 real-time RT-PCR assays.

Agarose gel electrophoresis of rRT-PCR reaction products from the Seg-1 real-time assays, was used to determine their specificity for BTV. A selection of non-BTV nucleic acids extracted from cell culture supernatants were tested using the optimised assay containing the eastern and western primer sets and both probes (Table 3). With the exception of BTV positive controls, all samples tested gave a ‘No Ct’ value. The absence of amplification was confirmed for selected samples of African Horse Sickness Virus (AHSV), Equine Encephalosis virus (EEV) and Epizootic Haemorrhagic Disease virus (EHDV).

3.5: Quantitative use of the assay

A time course experiment was performed to demonstrate the use of the optimised dual probe assay in a quantitative fashion. Approximately 120,000 BHK-21 cells per well were seeded into 24 well plates and allowed to reach 90% confluence at the time of infection. The cells were infected by replacing the growth media with 300 μ l per well of supernatant from a RSAvvvv/16 infected BHK cell culture that had been diluted 1:10 in unmodified GMEM. The virus (unmodified GMEM for mock infections) was allowed to adsorb to the cells for 30 minutes at RT $^{\circ}$ C before an additional 1ml of complete growth media was added to each well. At 0, 2, 4, 10 or 24 hours post infection the supernatant was discarded and the cell sheet harvested into 350 μ l RLT buffer containing β -2-mercaptoethanol (Materials and Methods, section 2.5.2, RNeasy Mini Kit, Qiagen).

The RNeasy animal-cell spin-protocol was used to purify total RNA from the cells, eluting RNA from the column using $2 \times 40 \mu$ l of RNase free water. A ten-fold dilution series of *in vitro* transcribed RNA (containing known copy numbers) was used as a standard curve for quantification. The standards and cellular RNA samples were tested using the RSA set of primers/RSA BTV 341-320 probe (Table 3.1) and the copy numbers in each sample calculated using the standard curve. The curve is highly linear ($R^2 = 0.997$) with a PCR efficiency approaching 100% (Figure 3.6). As expected, the Seg-1 copy number that was detected in the infected cells, increased with time post infection. At

'0 hours post infection' the input virus sample was detected with relatively little change observed between 0 and 2 hours post infection. However, between 2 and 4 hours post infection the copy number increased rapidly by 100 fold. From 4 hours onwards there was a steady increase in the copy number (Figure 3.6).

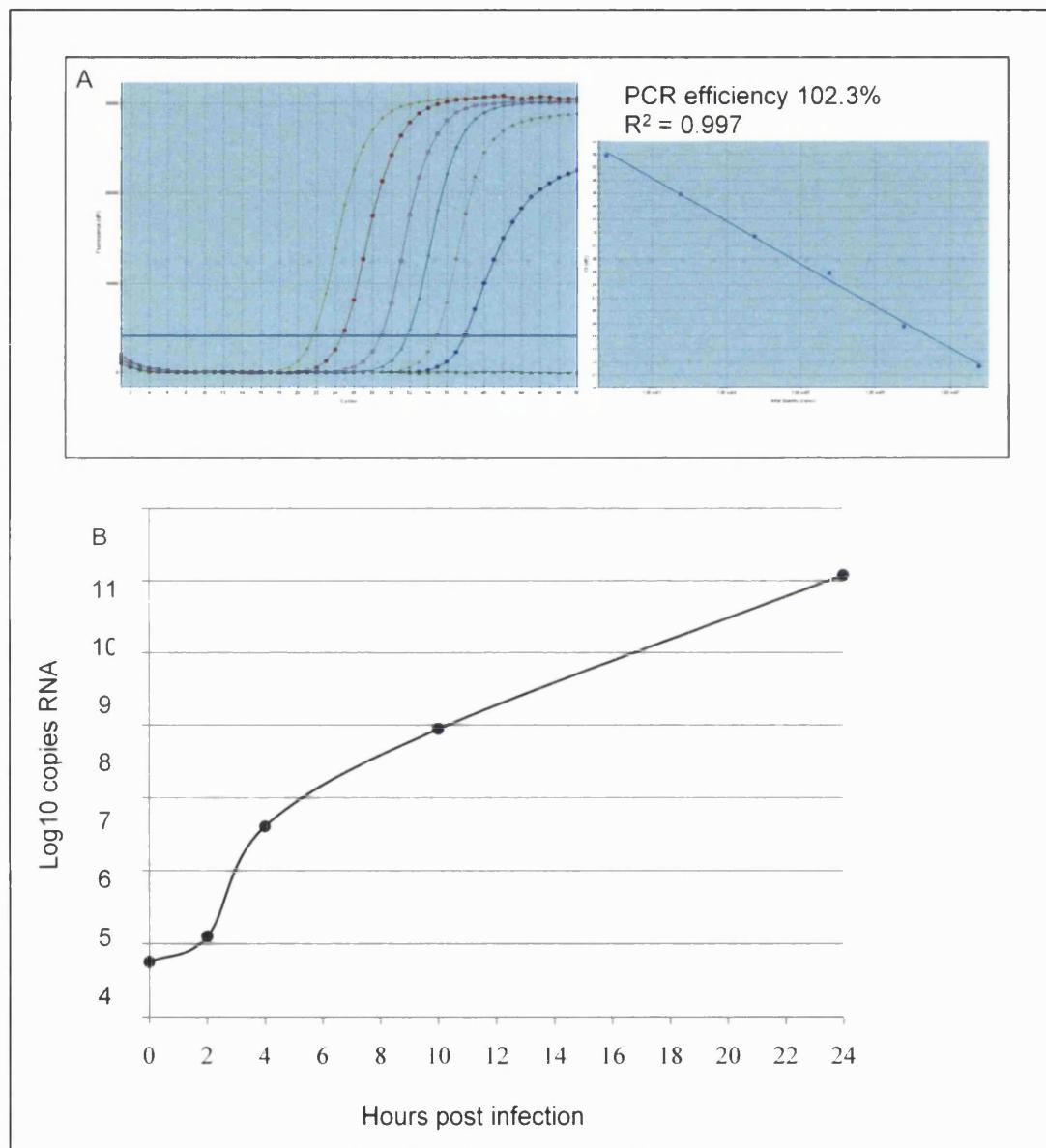


Figure 3. 6: Quantitative analysis of BTV RNA using the optimised rRT-PCR.
 BHK cells were infected with RSAvvvv/16 and samples were harvested at 0, 2, 4, 12 or 24 hours post infection. RNA was purified from cell lysates and analysed using the rRT-PCR reactions containing the RSA primers and RSA probe (Table 1). **A)** A standard curve of *in vitro* transcribed RNA allowed copy numbers to be calculated. The assay demonstrated high PCR efficiency with a slope R^2 of 0.997 indicating that quantitation would be reliable. **B)** RNA copy numbers were shown to increase over time with a dramatic rise observed between 2 and 4 hours post infection.

The dual probe assay (Shaw et al., 2007) was also used to monitor the level of BTV RNA throughout a time course of infection in cattle and sheep infected with the European strain of BTV-8. However, this study reported RNA levels by Ct value only without absolute quantitation of RNA copy numbers (Darpel et al., 2007).

3.6: Evolution of the assay

3.6.1: The Netherlands 2006

On the 17th August 2006, the Community Reference Laboratory (CRL) at Pirbright received blood samples from animals in the Netherlands for confirmation of BTV infection (Braakman, 2006). At that time, the CRL was using a ‘conventional’ RT-PCR and agarose gel based assay targeting to detect BTV genome segment 7 (Anthony et al., 2007). The remainder of the RNA sample, which had been extracted for use in the conventional RT-PCR, was also tested using the optimised dual probe real-time RT-PCR assay targeting Seg-1. Both assay systems gave identical results (three out of the six samples were positive). Two of the three PCR +ve samples were also +ve for antibodies to BTV VP7 by antibody ELISA. However, the remaining three samples were negative in all three assay systems.

These results support a number of significant conclusions:

- 1) The BTV strain involved could be detected by either conventional (Seg-7) or real-time (Seg-1) RT-PCR assays.
- 2) The conventional and real-time assays gave comparable results.
- 3) That BTV had arrived for the first time ever recorded in Northern Europe.

The Northern European BT outbreak which started in 2006, was subsequently shown to be caused by an African lineage of BTV-8 (Maan et al., 2008). During 2006, the outbreak spread to Germany, Belgium, the Netherlands, France and Luxemburg, then overwintered, re-emerging in May 2007 before rapidly escalating and spreading further across Europe (Saegerman et al., 2008). Due to the proximity of the outbreak, there was a fear that it would spread to the UK in 2007, with a possible need for high throughput and reliable screening of blood samples by RT-PCR. The closed tube format of ‘real-time’ assays is less prone to cross-contamination and is therefore better for large scale diagnostic testing. It was therefore decided to adapt the real-time Seg-1 rRT-PCR assay for robotic systems to increase handling speed and the numbers of samples that could be tested.

3.6.2: Robotic extraction of RNA

Before the Northern European outbreak of BT in 2006, the most significant ‘bottleneck’ in high throughput testing for BTV by rRT-PCR by the CRL, was the extraction of RNA from the original blood samples. This was performed manually using centrifugation and silica membrane columns (Anthony et al., 2007; Shaw et al., 2007). In an attempt to speed up this process, EDTA treated blood samples were also extracted using automated ‘robotic’ technology. 200 µl of each sample was added to 300 µl of Roche lysis/binding buffer. The resulting 500 µl was extracted on a Roche MagNA Pure LC robot using the ‘MagNA Pure LC Total Nucleic Acid Isolation kit’ (Roche, Mannheim, Germany) and the ‘Total NA External lysis.blk automated extraction programme’. The nucleic acid was eluted in 50 µl elution buffer (MagNA Pure LC Total Nucleic Acid Isolation kit, Roche). This protocol was used to extract nucleic acid from EDTA treated blood samples from the 2006 outbreak of BTV-8 in northern Europe, which included samples from an experimental infection of cattle and sheep that was conducted by members of the Vector Borne Diseases Programme at IAH Pirbright, using the BTV-8 strain from the Netherlands 2006 (NET2006/01 (Darpel et al., 2007)). Subsequent work by members of the CRL at IAH indicated that the Qiagen BioRobot Universal system was also suitable for the extraction of viral RNA from EDTA treated blood samples prior to testing them for BTV by r RT-PCR.

3.6.3: RNA denaturation

Denaturing dsRNA samples chemically using MMOH and β-2-mercaptoethanol, as described for the conventional assay targeting Seg-7 (Anthony et al., 2007; Mertens and Sangar, 1985) is both time consuming and poses a risk of cross-contamination. MMOH is also considered to be a dangerous chemical and its use has been banned in some countries. Heating RNA test-samples to 98 °C for five minutes (in a PCR reaction plate, using a thermal cycler) followed by rapid cooling on ice, was therefore evaluated as an alternative means of RNA denaturation prior to testing by rRT-PCR. A 10-fold titration series of RSAr04 cell culture derived virus (tissue culture supernatant) in uninfected ovine blood, was extracted using a Roche MagNA Pure LC robot, as described above. The viral RNA was subsequently detected down to a dilution of 10^{-5} (Figure 3.7). Heat denaturation gave lower Ct values (by approximately 2 Cts) than the MMOH treatment, indicating that it was slightly more efficient for denaturation of dsRNA templates.

Duplicate samples from a 10-fold dilution series of BTV-4 infected blood, were denatured using either MMOH or heating methods. Subsequent rRT-PCR assays targeting Seg-1, produced standard deviations ranging from 0.18 to 0.62, or 0.03 to 0.94 respectively. Both methods of denaturation gave PCR efficiencies close to 100%; (98.6% with MMOH, 97.8% with heat, Figure 3.7). In addition to demonstrating that the PCR efficiencies are very good, the good linearity is an indication that the robotic extraction is suitable for use with this assay.

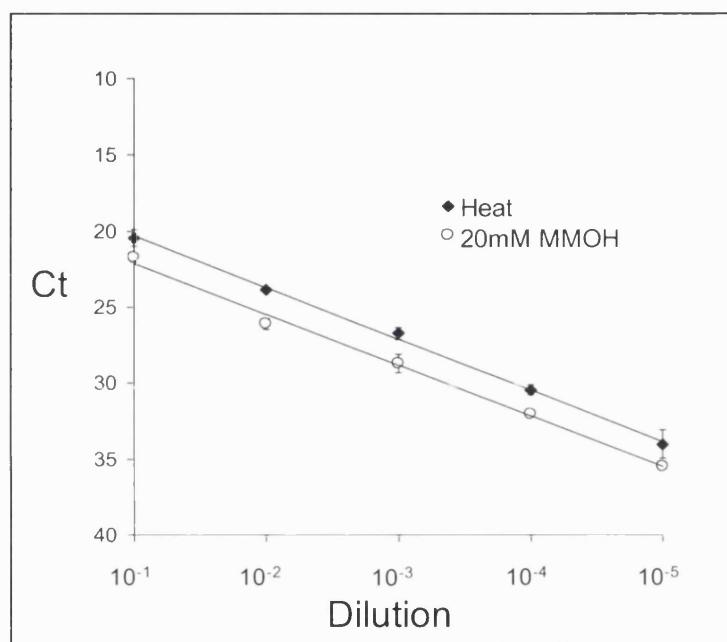


Figure 3.7: Standard detection curves, comparing BT RNA denaturation methods. BTV RNA samples were denatured either in 20mM methyl mercuric hydroxide (MMOH), or by heating for five minutes at 98°C and then rapid cooling on ice. The Seg-1 rRT-PCR efficiencies were almost identical using these methods (MMOH 98.6% / heating 97.8%) but heat denaturation resulted in Ct values that were approximately 2 cycles lower. Bars represent standard deviation.

3.6.4: Modification of the Seg-1 rRT-PCR probe sequence

Although all of the results obtained so far indicated that the ‘dual-probe assay’ was robust and would continue to demonstrate high sensitivity and specificity, there was an underlying concern that probe efficiency could be affected by the use of two overlapping probes, which might compete for the same site. It was therefore decided to test an alternative ‘single’ probe. Two probes were designed, which target different strands at the same site (called ‘RedunF’ and ‘RedunR’ which are reverse complements of each other). Two redundancies were included in these probes to reduce the number of

mismatches (Figure 3.8). The original RSA probe is capable of detecting western viruses but has a nucleotide mismatch with western viruses at its 3' terminal base, suggesting that binding of this nucleotide is unlikely to be critical for probe function. A mismatch at this point was therefore tolerated during the redesigning. A redundancy was included in order to avoid mismatches towards the upstream end of the probes, to guard against displacement (as opposed to cleavage) of the probe by the polymerase. Another redundancy was included at a position where several viruses contained a mismatch near the 3' end of the probe (Figure 3.8). These redundancies reduce the number of mismatches, compared to the available Seg-1 sequences, to zero (55%) or one (33%). Only four (12 %) of the sequences contained two mismatches, and no sequences contained 3 or more (Figure 3.8).

MIXED PROBE

	3'-TGGCACCTCACTTGAACCTAGGCCTCCT-5'	n=
BTV-2 (from AY154458)	G.....A...	2
BTV-2 (from L20508)	G.....	15
BTV-3 [NIG1982/06 (EF059721)]	G..A.....	1
BTV-8 [RSArrrr/08 (EF059727)]G.....A.....	2
BTV-9 [RSAvvv2/09]	G..A.....A.....	2
BTV-12 [NIG1982/09]	G..A.....C.....	1
BTV-22 [NIG1982/11 (EF059739)]	G.....C.....	1
BTV-24 [RSArrrr/24 (EF059741)]	G.....G.....	1
BTV-1 [GRE2001/02 (EF059720)]	...A.....A..	1
BTV-16 [RSAvvvv/16 (EF059734)]	5
BTV-21 [RSArrrr/21 (EF059738)]C.....A..	2

number with >1 change = 13

RedunR PROBE

	3'-CGG <u>Y</u> ACCTCACTTGAACCTAGG <u>Y</u> C-5'	n=
BTV-2 (from AY154458)	18
BTV-8 [RSArrrr/08 (EF059727)]	A.....G.....	2
BTV-9 [RSAvvv2/09]A.....	2
BTV-12 [NIG1982/09]C.....	2
BTV-24 [RSArrrr/24 (EF059741)]G.....	1
BTV-16 [RSAvvvv/16 (EF059734)]	A.....	6
BTV-21 [RSArrrr/21 (EF059738)]	A.....C....	2

number with >1 change = 4

Figure 3. 8: Alignments of Seg-1 probe sequences.

The ‘target-sequences’ for the ‘old’ and ‘new’ RT-PCR probes were aligned against different Seg-1 sequences. ‘n =’ refers to the number of sequences which match the sequence shown. (from the 33 that were available). The ‘mixed probe’ alignment summarises Figure 3 with the two original probes (Table 1 and Figure 3). The redundant bases (**Y** = C or T) are underlined in RedunR (nucleotides 342-320 according to GenBank accession number AY154458, RedunF is the reverse complement of RedunR). The viral sequences are displayed in the sense orientation.

Although the two redundancies ultimately result in four different version of each probe they all target the same footprint. It was considered possible that the probes

RedunF or RedunR might have a difference in affinity for their respective target strands. Therefore in order to determine whether their nucleotide composition had any effect on probe efficiency, the sense (RedunF) and anti-sense (RedunR) orientations of the redesigned probe were compared (Figure 7).

As amplification is largely determined by the primers used, rather than the probe, re-optimisation of the reaction conditions (primer concentrations, annealing etc.) was not thought to be necessary. The two probes in the original assay (0.5 µl of each - Shaw *et al-* 2007) were replaced by 0.5 µl of either RedunF, or RedunR. A selection of virus isolates were used for an initial performance comparison of the Redun probes, to the 'old' mixed probe assay (Table 3.4). The viruses chosen included RSArrrr/01 and RSAvvvv/16 (as representatives of western and eastern isolates respectively), isolates which had caused problems during development of the original assay (ITL2003/01 and RSAvvv2/09), and viruses which contained mismatches in the probe region (BTV-8 and BTV-12). ITL2003/01 and RSAvvv2/09 were diluted as before to avoid the inhibition encountered previously (section 3.4.2).

Sample	Shaw <i>et al</i>	RedunF	RedunR
RSArrrr/01 (BTV-1)	16.62	16.93	16.09
NIG1982/07 (BTV-8)	21.85	23.2	22.26
ITL2003/01 (BTV-9)	20.9	21.79	21.41
RSAvvv2/09 (BTV-9)	24	23.39	21.81
NIG1982/09 (BTV-12)	17.25	17.35	16.31
RSAvvvv/16 (BTV-16)	17.15	17.75	17.76
Neg	No Ct	No Ct	No Ct

Table 3. 4: Pilot test of Seg-1 rRT-PCR using redesigned probes with redundant bases.

Ct values are given for rRT-PCR assays with RNA samples from a selection of viruses. Each sample was tested in parallel, using the 'two-probe' assay, or the 'new' single probes (RedunF or RedunR) which have two 'redundant' bases. All RT-PCR reactions contained both the eastern and western primer sets and followed an identical protocol (see Materials and Methods).

The Ct values for the three different assays were largely similar, indicating an equivalent level of performance, showing that the redesign of the probes did not impact upon the overall efficiency of amplification from the primers. However, there was a large difference in the amplification plots. The plot for RedunF was almost identical to the original mixed probe assay, and reached a similar level of fluorescence in the plateau phase. In contrast, the RedunR probe consistently produced a steeper logarithmic phase

and reached a much higher plateau of fluorescence (Figure 3.9). The steeper curve suggests an increased release of fluorescence during each cycle which may indicate more efficient cleavage of the probe. These data indicate that the RedunR performed particularly well in this system.

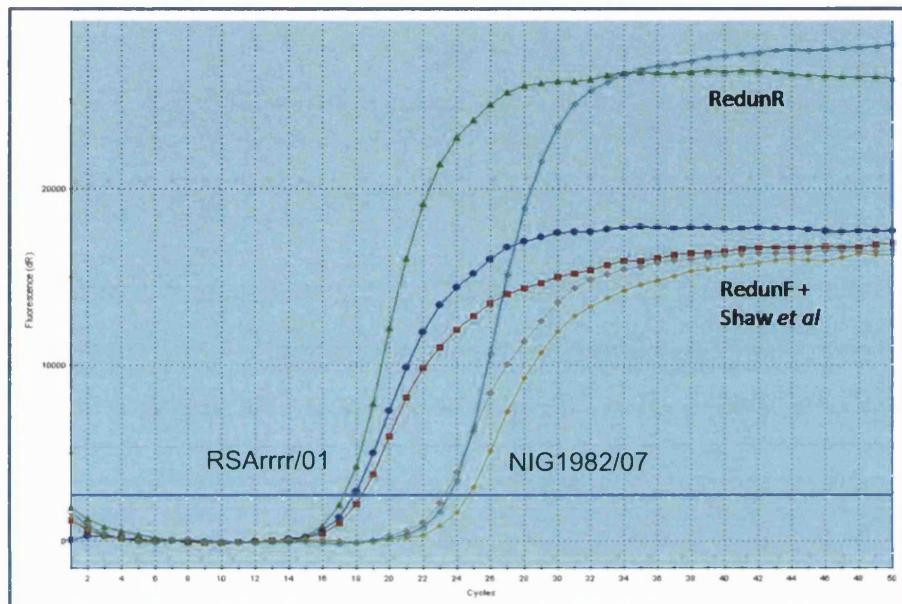


Figure 3. 9: Amplification plots for the redesigned and original Seg-1 rRT-PCR assays.

Amplification plots are shown for RNA samples from two BTV isolates, RSArrr/01 (BTV-1) and NIG1982/07 (BTV-8), which were tested in parallel using the original ‘two probes’ (Shaw *et al* 2007), RedunF or RedunR. The Ct values were largely similar, but the steepness of the curve and the plateau of fluorescence was much greater with the RedunR probe.

The largest difference in Ct value was for RSAvvv2/09 (the sample that gave the most problems during assay development due to probe sequence mismatches in conjunction with impure or excessively concentrated RNA), with values over 2 Ct lower than the Ct value obtained using the dual probe rRT-PCR assay, indicating that the RedunR probe may be less sensitive to the hindrance of excess template or contaminants. The amplification plots produced by all three assays for RSAvvv2/09 were more ‘linear’ than for other samples but overall the RedunR probe again demonstrated curves indicative of more efficient amplification (Figure 3.10).

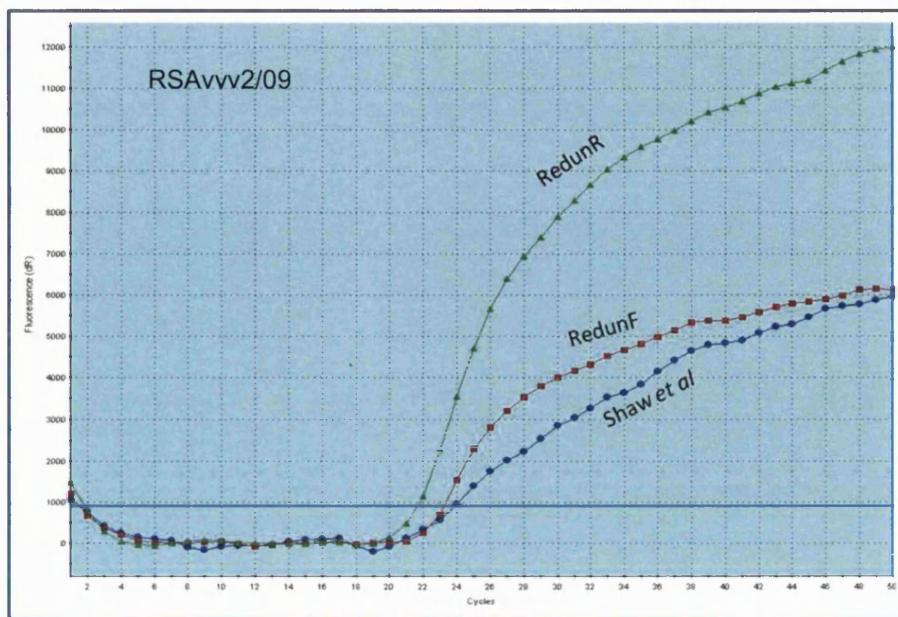


Figure 3. 10: Amplification plots for RSAvv2/09 with different rRT-PCR probes.

The impact of different probe sequences on cDNA amplification was assessed. Purified RSAvv2/09 RNA was tested in parallel, in each case using identical reaction mixtures and conditions, with the exception of the probe incorporated into the mix (as indicated). Both of the probes incorporating redundancies gave improved amplification plots compared with the original dual probe assay (Shaw et al 2007). However, the RedunR probe outperformed both the RedunR probe and the Shaw et al assays, producing a lower Ct value, steeper curve and higher peak of fluorescence.

The sensitivity and specificity of the RedunR modified probe was tested by the CRL at Pirbright using automated extractions (data not available). In line with the initial experiments described here, the assay using RedunR outperformed the original assay containing the two overlapping probes. The RedunR assay was also used to analyse RNA from the same animal experiment (Darpel et al., 2007) that had previously been tested using the original assay, consistently producing equivalent or lower Ct values. This confirmed that use of the RedunR probe maintained or improved the overall performance of the assay.

After the start of the BTV-8 outbreak in Northern Europe, many countries quickly implemented real-time RT-PCR assays for detection and diagnosis of BTV. Several national laboratories have adopted the assay described here, based upon the published assay (Shaw et al., 2007). Many laboratories have successfully used these primers and probes, based on different reaction chemistries and using different rRT-PCR equipment,

indicating that the assay is sufficiently robust to tolerate these variations from the original published protocol.

The redesigned RedunR probe sequence was also disseminated under confidentiality agreements to laboratories who requested details of the modified assay. However, the RedunR probe modification has not yet been published because it is the subject of a commercialisation agreement with Qiagen (the '*cador* BTV RT-PCR kit').

3.7: Commercialisation

As a result of the massive European outbreak of BTV-8 starting in 2006, thousands of samples needed to be tested for diagnosis, surveillance and import / export testing. In view of the ongoing vaccination campaigns in Europe, the serological methods (primarily competition ELISA) that were previously used to detect BTV-specific antibodies for diagnosis are no longer effective. However, animals vaccinated with the inactivated BTV-8 vaccine do not give a positive signal by rRT-PCR, and the demand for BTV testing represented an attractive commercial opportunity for companies looking to expand the list of pathogens in their molecular diagnostics repertoire.

The original published assay containing two probes (Shaw et al 2007) was formulated into a kit by Applied Biosystems and was made available as the VetMAX™-Plus Multiplex One Step RT-PCR Kit. Qiagen approached the IAH with the aim of developing its own BTV RT-PCR kit. It was agreed that the modified probe sequence would be used in conjunction with the published primers (Shaw et al., 2007) and using Qiagen chemistry (the '*cador* BTV RT-PCR kit') (Figure 3.11).

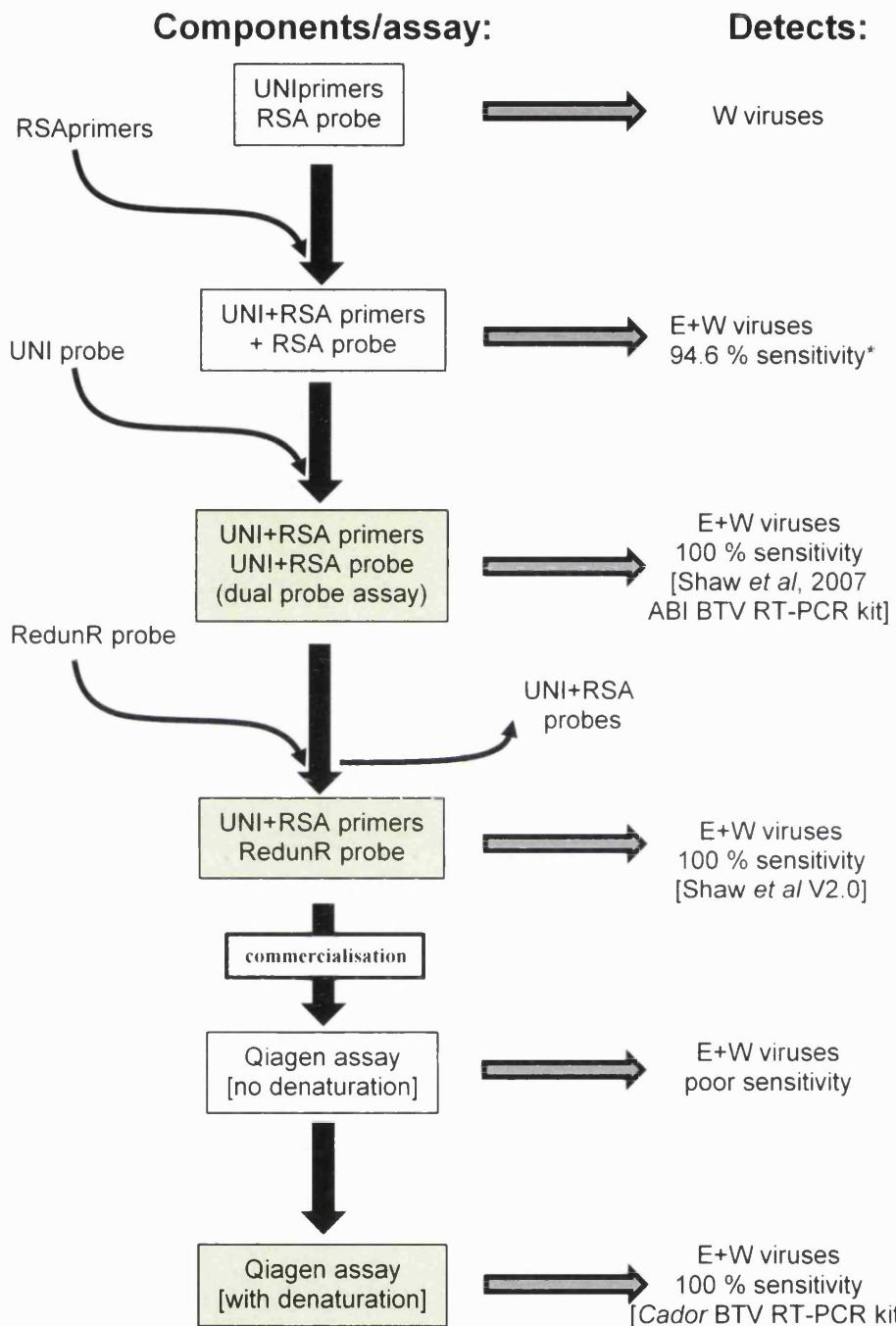


Figure 3. 11: Evolution of the pan-BTV real-time RT-PCR assay.

The assay started with one set of primers and one probe was capable of detecting western viruses. The successive addition of a second (RSA) set of primers and a second probe (UNI) allowed 100% diagnostic sensitivity. The replacement of both probes with RedunR alone resulted in an assay with enhanced fluorescence characteristics. This assay was commercialised, resulting in the *Cador* BTV RT-PCR kit. The three major assays are highlighted by green shading.

* Sensitivity refers to the diagnostic sensitivity of the assay.

3.7.1: Pilot testing of the kit

Pre-production ‘test kits’ and a protocol of the assay, using the modified probe, were provided by Qiagen for evaluation in comparison to the modified version of the original assay (here referred to as the Shaw *et al* V2.0 assay). The Qiagen ‘*cador* BTV RT-PCR kit’ comprises a ‘mastermix’, a tube of magnesium solution, a tube of internal positive control template (IPC) and a tube of *in vitro* synthesised BTV dsRNA template, as an exogenous positive control. The mastermix contains all of the components (with the exception of magnesium) required for the reverse transcription and PCR reactions. Amplification of any BTV-specific sequences is detected in the FAM channel of real-time RT-PCR machines, whereas the IPC amplification is detected in the HEX/VIC/JOE channel.

The Qiagen assay was initially used to test samples from the European BTV diagnostic laboratories ring trial in 2007 (Batten *et al.*, 2008a). The BTV Ring Trial included a ‘blind’ set of samples (identified only by code) that were assembled and sent in parallel to each participating laboratory. The laboratories each test the samples and the results are collated to gauge the proficiency and uniformity of BTV diagnosis between laboratories (Batten *et al.*, 2008a; Batten *et al.*, 2009a). The Qiagen assay was tested by the CRL at Pirbright and the results are presented in Table 3.5.

Sample	Shaw <i>et al</i> V2.0	Qiagen
A163/08 3 (Dutch BTV-6)	29.62	22.47
BTv 6 REF	16.63	8.83
3011 (BTv-8)	30.80	29.90
3012 (BTv-2)	28.99	19.67
3013 (Neg)	No Ct	No Ct
3014 (BTv-4)	29.07	19.15
3015 (BTv-1)	23.87	17.71
3016 (Neg)	No Ct	No Ct
3017 (BTv-8 and -1)	25.48	19.10
3018 (BTv-16)	27.24	18.76
3019 (BTv-8)	32.13	No Ct
3020 (BTv-1)	28.62	No Ct
BTv POSITIVE CONTROL	28.54	23.93
IPC ^a	N/A	18.25
BTv Qiagen POS ^b	N/A	14.80

Table 3.5: Initial testing of the Qiagen ‘*cador* BTV RT-PCR kit’.

The initial test-version of the ‘*cador* BTV RT-PCR kit’ was evaluated using test samples from the 2007 BTV ring trial (Batten *et al.*, 2008a). The Ct values shown are for RNA samples that had been robotically extracted and tested using either the published Shaw *et al* V2.0 assay, or according to the protocol provided by Qiagen. With the exception of two weak positive samples all of the samples that were positive by the published assay were also positive using the Qiagen assay.

^a The IPC is a component of the Qiagen assay only and therefore the Shaw *et al* V2.0 assay has no result

^b The BTV Qiagen POS control refers to the positive control provided with the Qiagen assay and therefore the Shaw *et al* assay has no result

With the exception of samples 3019 and 3020, there was a good overall correlation between the original assay, and the Qiagen kit (Table 3.5). Although other weak samples were detected by the Qiagen assay (e.g. 3011), these data suggest that the Qiagen assay may perform poorly with weak samples. Using the Shaw *et al* (2007) assay as the ‘gold standard’, the diagnostic sensitivity and specificity values (excluding the IPC and BTV Q control) were 82% and 100% respectively (n = 13) for the Qiagen assay. A wider range of ‘weak +ve’ diagnostic samples that were previously identified using the Shaw *et al* V2.0 assay, was subsequently tested using the Qiagen assay (Table 3.6).

Sample	Species	Shaw <i>et al</i> V2.0	Qiagen (BTV)	Qiagen (IPC)
A182/08	Bovine	31.48	No Ct	17.83
B1369/08 05	Bovine	28.48	No Ct	17.11
B1398/08 01	Bovine	24.33	18.22	17.24
B1398/08 02	Bovine	24.22	20.05	17.01
B1398/08 03	Bovine	24.94	21.18	17.13
B1398/08 04	Bovine	31.07	No Ct	16.43
B1398/08 05	Bovine	No Ct	No Ct	17.3
B1398/08 06	Bovine	No Ct	20.87	17.53
B1476/08 02	Ovine	No Ct	No Ct	17.72
B1505/08 01	Bovine	No Ct	No Ct	16.45
B1505/08 02	Bovine	No Ct	No Ct	17.09
B1524/08 01	Bovine	32.2	No Ct	17.34
B1524/08 02	Bovine	30.25	No Ct	17.11
B1524/08 05	Bovine	33.39	No Ct	17.45
B1524/08 07	Bovine	31.29	No Ct	17.68
B1524/08 09	Bovine	29.75	No Ct	17.27
B1524/08 16	Bovine	34.66	No Ct	16.59
B1524/08 19	Bovine	33.38	No Ct	16.95
B1524/08 20	Bovine	32.96	No Ct	16.19
B1524/08 24	Bovine	32.63	No Ct	17.88
B1524/08 26	Bovine	34.73	No Ct	17.4
B1524/08 31	Bovine	34.55	No Ct	17.25
B1534/08 14	Bovine	38.68	No Ct	16.52
B1602/08 02	Bovine	28.36	No Ct	17.04
B1602/08 03	Bovine	27.66	33.29	18.3
B1602/08 06	Bovine	31.87	No Ct	17.18
B1602/08 07	Bovine	29.55	No Ct	17.18
B1602/08 08	Bovine	29.3	No Ct	16.95
BTV neg 161007	Bovine	No Ct	No Ct	17.43
BTV POS 141108	Bovine	27.2	21.03	17.05
BTV Qiagen POS	N/A	N/A	14.74	16.94
BTV Qiagen POS	N/A	N/A	14.91	17.08
No Template Control	N/A	N/A	No Ct	16.94

Table 3.6: Testing of the Qiagen ‘*cador* BTV RT-PCR kit’ with weak +ve samples.

A collection of RNA samples extracted (robotically) from EDTA treated blood was tested using the Qiagen ‘*cador* BTV RT-PCR kit’. 19 out of 23 samples that were weak positives using the Shaw *et al* V2.0 assay were negative using the Qiagen assay. However, sample B1398/08 06 was negative using the Shaw *et al* V2.0 assay, but positive using the Qiagen assay.

82.6 % of RNA samples (extracted from blood) that were weak-positives by the Shaw *et al* V2.0 assay, produced 'No Ct' using the Qiagen assay, indicating poor sensitivity. At this stage of development the differences between the qiaegen kit and the original assay were:

- the RT-PCR chemistry/reagents
- the Qiagen assay contains an IPC
- the Qiagen assay setup did not incorporate denaturation of the RNA sample

Altering the chemistry was not an option for a commercial company. It was conceivable that the IPC may reduce the sensitivity of the assay due to competition with the BTV-specific amplification for reagents. To test this hypothesis several samples were tested using the kit, either with or without the IPC included in the mastermix.

Sample	Species	Shaw <i>et al</i> V2.0	Qiagen (BTV)	Qiagen (IPC)
B1369/08 5	Bovine	28.48	+IPC -IPC	28.73 No Ct
B1398/08 1	Bovine	24.33	+IPC -IPC	23.68 19.46
B1398/08 2	Bovine	24.22	+IPC -IPC	22.39 19.61
B1398/08 3	Bovine	24.94	+IPC -IPC	20.87 22.3
B1398/08 4	Bovine	31.07	+IPC -IPC	No Ct No Ct
B1398/08 5	Bovine	No Ct	+IPC -IPC	27.89 25.36
B1398/08 6	Bovine	No Ct	+IPC -IPC	21.83 21.23
BTV Qiagen Pos	N/A	N/A	+IPC	15.09
			+IPC	15.4
BTV Qiagen Pos	N/A	N/A	-IPC	15.01
			-IPC	15.17

Table 3. 7: Influence of internal positive controls (IPC) on sensitivity of the Qiagen assay.

Seven bovine blood samples positive for BTV-8 Each RNA sample (see Table 6) was tested in parallel using a Qiagen mastermix with (+IPC) or without (-IPC) the IPC included in the mastermix. Although the Qiagen assay failed to detect B1398/08 4, the Qiagen assay found two samples to be positive which had tested negative using the Shaw *et al* V2.0 assay.

The results indicate that the presence of the IPC can in some cases have an inhibitory effect upon the sensitivity of the assay (e.g. B1369/08 5 and B1398/08 1 - Table 3.7), suggesting that this may have limited detection of weak samples. Although there was an instance where the Qiagen assay failed to detect a weak positive sample (B1398/08 4), there were again two samples (B1398/08 5 and B1398/08 6) which were negative when tested by the Shaw *et al* V2.0 assay, but positive when tested using the Qiagen assay.

It was considered possible that the IPC template was too concentrated in the final RT-PCR reaction mix, and might be out-competing the target BTV Seg-1 reaction. Qiagen supplied another sample kit with three different IPC template dilutions. These were tested against RSAvvvv/16, the Qiagen BTV control provided in the kit, and a no template control (water), the results of which are shown in Table 3.8.

Sample	IPC template	Qiagen (FAM)	Qiagen (HEX)
RSAvvvv/16 IPC	IPC	10.77 / 11.41	16.12 / 15.76
	IPC-1	13.94 / 12.64	No Ct / No Ct
	IPC-2	14.86 / 13.04	No Ct / No Ct
BTV Qiagen POS IPC	IPC	15.06 / 15.17	14.78 / 14.78
	IPC-1	14.85 / 15.11	17.22 / 17.3
	IPC-2	14.78 / 14.84	19.79 / 17.12
water IPC	IPC	No Ct / No Ct	14.34 / 14.5
	IPC-1	No Ct / No Ct	17.07 / 17.2
	IPC-2	No Ct / No Ct	20.46 / 19.57

Table 3. 8: The effect of different IPC dilutions upon Qiagen assay performance.

Three ‘Internal Positive Control’ concentrations (IPC-1, IPC-2 and IPC-3) were tested against BTV RNA, and/ or the internal +ve control, in duplicate in reactions that were otherwise identical. ‘IPC’ was the initial IPC template concentration, ‘IPC-1’ is 10 times weaker than ‘IPC’, and ‘IPC-2’ is 100 times weaker than ‘IPC’. The FAM signal refers to that obtained for the detection of BTV, whereas the HEX signal refers to the detection of the IPC template in the reaction. The IPC concentration had little effect on the results obtained.

The results showed that the concentration of the IPC had very little impact upon the performance of the assay. It was therefore decided to use the IPC at the original concentration.

The remaining difference between the Shaw *et al* assay and the Qiagen assay was the absence of a denaturation step in the Qiagen protocol.

3.7.2: The effect of denaturation: late vs. early infection

The early version of the Qiagen assay did not include a denaturation step prior to RT. To investigate the possibility that denaturation might improve the assay, weak BTV-8 +ve samples originally submitted to the CRL by Belgium, were tested using the assay, both without denaturation and with a heat denaturation step identical to the Shaw et al (2007) assay.

Sample	+ denaturation		- denaturation		
	Qiagen (BTV)	Qiagen (IPC)	Qiagen (BTV)	Qiagen (IPC)	Shaw et al V2.0
Belgium BTV-11 1	No Ct	14.47	No Ct	15.03	40.13
Belgium BTV-11 2	20.98	14.37	No Ct	14.71	32.99
Belgium BTV-11 3	19.8	14.88	No Ct	14.31	32.97
Belgium BTV-11 4	19.65	15.2	No Ct	14.93	31.94
Belgium BTV-11 6	20.5	14.85	No Ct	14.76	32.35

Table 3. 9: The effect of sample denaturation on BTV RNA detection by the Qiagen assay.

Weak +ve BTV-RNA samples (extracted robotically from blood from Belgium) were tested, with or without denaturation. No samples were positive in the absence of denaturation but four of the five samples were detected when the samples were denatured by heating to 98 °C for five minutes. However, one very weak sample (with a Ct of 40.13) using the Shaw *et al* V2.0 assay, was still negative using the Qiagen assay, even after denaturation.

Heat denaturation had a dramatic impact upon the sensitivity of the Qiagen assay (Table 3.9). In the absence of a denaturation step, none of the weak +ve samples were detected using the Qiagen assay. In contrast, four of the five samples were detected by the Qiagen assay when the samples were first denatured by heating. The remaining sample (Belgium BTV-11 1) represents a sample at the limit of detection of the Shaw *et al* assay. These data demonstrate that a denaturation step is required for the detection of weak samples.

3.7.3: Broader testing of samples – assay sensitivity

A characteristic of the assay published by Shaw *et al* (2007) is its ability to reliably detect all 24 serotypes of BTV, while retaining specificity for the BTV serogroup. To confirm that this ability had been maintained during commercialisation, the Qiagen assay was tested against reference strains of the 24 BTV serotypes as well as two serotypes of EHDV, one serotype of AHSV and a homogenate of bovine epithelium

prepared according to standard procedures (Ferris and Dawson, 1988). Samples were extracted using the MagNA Pure and tested according to the optimised protocol.

Sample	Qiagen (BTV)	Qiagen (IPC)
RSArrr/01	18.42	19.55
RSArrr/02	17.41	19.48
RSArrr/03	21.18	19.42
RSArrr/04	16.88	19.2
RSArrr/05	19.55	19.5
RSArrr/06	18.49	19.19
RSArrr/07	20.34	19.13
RSArrr/08	20.28	19.48
RSArrr/09	20.35	19.45
RSArrr/10	18.71	19.28
RSArrr/11	19.4	19.22
RSArrr/12	18.73	19.15
RSArrr/13	20.47	19.24
RSArrr/14	18.44	19.31
RSArrr/15	22.25	18.96
RSArrr/16	22.69	19.62
RSArrr/17	19.88	19.47
RSArrr/18	19.84	19.22
RSArrr/19	19.74	19.09
RSArrr/20	22.63	19.4
RSArrr/21	21.31	19.2
RSArrr/22	21.14	19.2
RSArrr/23	20.67	18.79
RSArrr/24	20.39	19.42
Qiagen BTV POS	15.53/15.17	19.25/19.07
RSArrah/01 (AHSV-1)	No Ct/No Ct	19.52/19.41
EHDV-1 USA_01	No Ct/No Ct	18.94/18.97
EHDV Ibaraki	No Ct/No Ct	19.29/19.22
Uninfected Bovine epithelium	No Ct/No Ct	19.14/19.47
NTC	No Ct/No Ct	19.03/18.78

Table 3. 10 Detection of all 24 serotypes using the Qiagen assay (with sample denaturation).

RNA was extracted from BHK cell culture material infected with isolates of the 24 reference strains of bluetongue virus, the closely related orbiviruses African horse sickness virus (AHSV) and epizootic haemorrhagic disease virus (EHDV), and a homogenate of bovine epithelium using the MagNA Pure robot and tested using the Qiagen BTV RNA detection kit, with denaturation.

The Qiagen assay in combination with a denaturation step efficiently detected the reference strains of the 24 BTV serotypes (Table 3.10). The IPC was detected in each of the samples indicating a lack of any inhibition. Non-BTV templates gave No Ct results indicating that the assay is specific for BTV alone and did not cross-react with closely related viruses or host nucleic acid.

A more extensive range of samples was subsequently tested to further evaluate the ‘diagnostic sensitivity’ of the assay (Table 3.11).

Sample	Qiagen (BTV)	Qiagen (IPC)
6547 (BTV)	9.66	15.61
34678 (BTV)	7.02	13.75
BTV-1 IND1992/02 [2.3µl]	15.77	14.03
BTV-1 IND2001/01	14.29	14
BTV-1 AUS1981/01	20.03	14.87
BTV-1 GRE2001/04	14.09	14.31
BTV-1 GRE R22/01E	13.18	14.09
BTV-1 GRE2001/03	10.23	14.93
BTV-1 GRE2001/10	17.58	15.28
BTV-1 IND E	16.8	14.53
BTV-1 IND(D) [3.7µl]	17.85	14.81
BTV-1 NIG1982/01 [2.6µl]	9.43	13.68
BTV-1 SUD1987/01 [1µl]	8.75	14.03
BTV-2 FRA2001/06	9.91	14.11
BTV-2 SUD1985/01 [8.6ul]	12.13	14.68
BTV-2 IND1982/01	19.3	14.53
BTV-2 ITL2002/02	8.86	21.14
BTV-2 ITL2002/03	8.97	No Ct
BTV-2 ITL2002/04	8.92	No Ct
BTV-2 ITL2002/05	8.86	No Ct
BTV-2 ITL2002/06	9.81	9.53
BTV-2 ITL2002/07	9.08	No Ct
BTV-2 NIG1982/02	10.05	14.06
BTV-2 NIG N.421	5.96	12.72
BTV-2 SAD2002/01	9.91	13.49
BTV-2 SAD2001/04	18.86	13.2
BTV-2 SAD2002/02	10.28	14.41
BTV-2 SAD2001/05	16.59	14.61
BTV-2 SAD2001/01 [0.8µl]	10.89	13.98
BTV-2 SAD2001/02	16.54	14.77
BTV-2 SAD2001/03	11.31	13.97
BTV-2 SAD2002/03	16.59	14.48
BTV-2 SAD2001/06	15.02	13.93
BTV-2 SAD2001/07	19.59	13.98
BTV-2 TUN2000/01	19.31	14.19
BTV-2 RSAvvv2/02	16.99	14.89
BTV-2 ZIM2003/01	9.12	No Ct
BTV-23 IND1988/03	10.32	13.93
BTV-3 RSArrrr/03	5.82	13.6
BTV-3 NIG1982/06	19.91	15.3

BTM-3 ZIM2002/02	5.93	12.19
BTM-3 ZIM2002/01	12.61	14.96
BTM-4 RSArrrr/04	18.51	14.7
BTM-4 ARG2002/04	7.14	13.62
BTM-4 ARG2002/02	11.91	14.73
BTM-4 ARG2002/03	10.33	12.86
BTM-4 ARG2002/01	5.28	13.11
BTM-4 GRE1999/02	9.71	18.7
BTM-4 GRE1999/03	9.49	No Ct
BTM-4 GRE1999/05	9.29	No Ct
BTM-4 GRE1999/11	9.36	19.83
BTM-4 GRE2000/03	5.53	13.71
BTM-4 GRE2000/06	10.14	14.95
BTM-4 SUD1983/01	15.95	14.28
BTM-4 TUR1978/01	6.03	13.04
BTM-4 TURvvvv/04	8.95	No Ct
BTM-4 RSAvvv7/04	7.18	13.72
BTM-4 RSAvvv3/04	5.85	13.03
BTM-4 RSAvvv5/04	7.12	12.82
BTM-5 RSArrrr/05	5.53	11.89
BTM-5 CAR1982/02	5.75	12.92
BTM-4 GRE1999/10	9.54	18
BTM-5 NIG1982/04	5.17	15.18
BTM-5 NIG1982/03	10.36	12.22
BTM-6 RSArrrr/06	9.99	10.9
BTM-7 RSArrrr/07	7.7	13.11
BTM-8 RSArrrr/08	6.91	13.43
BTM-8 KEN----/01	6.2	14.39
BTM-8 NIG1982/07	10.87	12.92
BTM-9 RSArrrr/09	9.62	22.5
BTM-9 BOS2002/01	5.97	13.59
BTM-9 BOS2002/02	7.72	13.95
BTM-9 BOS2002/03	18.28	14.67
BTM-9 BUL1999/01	12.32	14.58
BTM-9 GRE1999/06	10.27	14.4
BTM-9 GRE2000/02	10.19	No Ct
BTM-9 ITL2002/01	7.01	14.01
BTM-9 RSAvvvv/09	9.67	19.47
BTM-9 RSAvvv9/09	10.45	13.09
BTM-9 RSAvvv2/09	12.85	14.24
BTM-9 TUR2000/11	10.46	16.08
BTM-9 TUR2000/12 [4.2μ]	8.75	13.96
BTM-9 TUR2000/03	7.27	12.63
BTM-9 TUR2000/05	5.74	13.77
NEG	No Ct	13.89
NEG	No Ct	14.89

Qiagen BTV pos	14.59	14.63
Qiagen BTV pos	13.76	14.65

Table 3. 11: Extensive testing of the Qiagen commercial assay.

RNA samples which had been extracted from cell culture supernatants using the QiaAmp viral RNA kit which had previously been tested during evaluation of the original rRT-PCR assay (Shaw *et al* (2007)) were re-tested using the Qiagen assay and incorporating a denaturation step. Every BTV sample gave a positive Ct value but in nine samples the IPC was not consistently amplified. Square brackets [x] refer to the volume of RNA input if <10µl.

Sample	Run 1		Run 2 (1:50)		Run 3 (1:2000)	
	Qiagen (BTV)	Qiagen (IPC)	Qiagen (BTV)	Qiagen (IPC)	Qiagen (BTV)	Qiagen (IPC)
1SA 19/8 (BTV)	No Ct	No Ct	12.91	No Ct	9.35	19.7
1SA/Vacc Chris (BTV)	No Ct	No Ct	11.36	No Ct	10.02	14.5
BTB-2 FRA2001/03	No Ct	No Ct	9.24	No Ct	8.64	13.34
BTB-2 ITL2002/01	No Ct	No Ct	9.05	13.03	16.35	14.69
BTB-9 VACC	No Ct	No Ct	9.94	No Ct	6.15	14.39

Table 3. 12: Three RT-PCR runs demonstrating the diluting-out of RT-PCR inhibition.

When tested undiluted, a selection of samples gave 'No Ct' for both the BTV and IPC results indicating the presence of RT-PCR inhibition. Dilution of the samples resulted in increasingly efficient detection of the BTV target as well as the IPC.

During testing it was observed that five samples were negative for BTV (Table 3.11). However, the IPC was also negative, indicating inhibition of the reaction. These were the same samples which demonstrated inhibition, possibly due to an excessive concentration of RNA. Dilution of the samples 1:50 allowed the detection of all five BTV samples, although in most cases (4/5) the IPCs still failed to amplify (Table 3.12). The suggestion that concentrated samples are a source of inhibition is supported by the 'No Ct' values obtained for the IPC in Run 2 (samples diluted 1:50) but positive values / detection in Run 3. The Ct values were also lower for the BTV RNA samples when they were diluted to 1:2000 compared to 1:50.

3.7.4: Selected blood derived RNA samples – clinical sensitivity

In order to be 'effective' it is important that routine diagnostic assays can be used with 'normal' diagnostic samples. Blood samples are relatively easy and non-invasive/damaging to obtain. The clinical sensitivity of the Qiagen assay (i.e. its ability to detect BTV RNA in whole-blood samples) was therefore assessed.

A range of BTV +ve RNA samples were available, which had been extracted from ovine or bovine EDTA treated whole blood and tested using the Shaw *et al* V2.0

assay. A selection of these was also tested using the Qiagen assay, incorporating a denaturation step. Two volumes of RNA were tested in each case to determine whether halving the input volume would affect the assay sensitivity. Sixteen of the samples were positive with both volumes of RNA (Table 3.13). Four samples gave 'No Ct' results when the reaction contained 10 µl of RNA. However, when only 5 µl of RNA was used, one of these samples gave a positive Ct value (Table 3.13). These data may suggest that the RNA contains impurities, although insufficiently inhibitory as to eliminate amplification of the IPC.

Sample	10µl		5µl	
	Qiagen (BTV)	Qiagen (IPC)	Qiagen (BTV)	Qiagen (IPC)
BTV A139/7/3 Switzerland	11.48	17.27	13.39	16.09
BTV A111/7/1 Gibraltar	10.66	17.97	12.78	16.59
BTV A33/08/06 Czек	16.3	17.06	17.48	16.17
BTV A111/7/11 Gibraltar	16.81	17.96	19.81	16.25
BTV A160/08/8 Libya	16.63	17.42	20.46	16.64
BTV A160/08/10 Libya	19.09	17.03	21.84	16.57
BTV A160/08/18 Libya	14.26	15.87	16.11	16.7
BTV A140/08/39 Turkey	No Ct	18.84	No Ct	17.64
BTV B377/07/4 UK	15.21	18.76	15.34	17.21
BTV B412/7/1 14/11/7	9.64	16.51	11	15.87
BTV B484/08/1 UK	16.23	18.52	17.25	17.58
BTV BH287/08/60	16.43	18.3	15.83	17.01
BTV B1410/08/1 UK	No Ct	16.33	No Ct	16.99
BTV B1340/08/1 UK	15.99	17.58	16.93	16.38
BTV B446/08/01 UK	15.62	18.07	16.31	17.28
BTV B1403/08/1	No Ct	17.65	28.66	16.01
BTV B1394/08/3 UK	22.45	17.63	23.46	16.4
BTV B539/7/1	12.19	16.97	13.49	16.39
BTV B754/08/1 UK	18.87	18.03	20.3	16.99
BTV B1409/08/2	No Ct	17.71	No Ct	16.74
Qiagen pos	14.83	17.09	15.79	16.19
Qiagen pos	14.46	17.28	15.87	16.39
NTC	No Ct	17.34	No Ct	16.83
NTC	No Ct	17.34	No Ct	16.7

Table 3. 13 Clinical sensitivity of the Qiagen BTV rRT-PCR assay.

A collection of BTV +ve RNA samples that had been extracted from EDTA treated blood samples were tested using the Qiagen assay. The sample names are codes provided for field samples by the CRL at Pirbright. Two volumes of RNA were tested to determine whether excess RNA impacted upon the assay performance. One sample was positive when 5 as opposed to 10 µl of RNA was used, possibly indicating a level of inhibition in the sample.

3.7.5: Testing Libyan diagnostic samples by rRT-PCR

A batch of 11 blood samples was received from Libya for BTV diagnosis by the CRL. These samples were subsequently ‘typed’ as a western strain of BTV-9. RNA was extracted from the blood by robot and tested using both the Shaw *et al* V2.0 assay and using the new Qiagen kit (with denaturation).

Sample	Qiagen (BTV)	Qiagen (IPC)	Shaw <i>et al</i>
A160/8/1	17.12	18.82	28
A160/8/2	11.51	18.32	27
A160/8/4	14.05	18.99	26
A160/8/5	11.96	18.89	21
A160/8/7	No Ct	20.73	No Ct
A160/8/9	15.41	20.59	22
A160/8/11	12.6	21.24	22
A160/8/12	7.64	No Ct	16
A160/8/13	14.89	20.38	25
A160/8/16	11.4	No Ct	22
A160/8/17	12.18	No Ct	19
A160/8/19	15.89	18.76	26
Qiagen BTV POS	15.35	19.93	N/A
NTC	No Ct	21.25	No Ct

Table 3. 14: Comparison of the Qiagen and original double probe assay with Libyan diagnostic samples.

A comparison was made of the Shaw *et al* V2.0 assay and the Qiagen assay with denaturation for analysis of Libyan diagnostic blood samples. Eleven out of twelve blood samples were positive for BTV RNA and one negative, according to both assays. This represents 100 % agreement between the assays using clinical samples, although the CT values obtained were significantly different. Three of the Qiagen IPCs gave ‘No Ct’.

The two assays gave identical diagnostic results, although with significant differences in Ct values (Table 3.14). Eleven of the samples were positive for BTV with the remaining sample negative. In three cases, where the Ct values from the Qiagen BTV assay were numerically low (<12.5. i.e. a strong signal) the IPC gave ‘No Ct’ results.

3.8: Discussion

This study demonstrates the suitability of Seg-1 as a diagnostic target for the sensitive detection of RNA from isolates representing any of the 24 BTV serotypes, as well as different topotypes and strains. The sequences of Seg-1 from different BTV strains can be divided into two distinct groups, representing geographically distinct clusters: an eastern topotype (including isolates from the Middle-east, Asia and

Australasia) and a western topotype (including isolates from Africa and the Americas). Differences in the Seg-1 sequence made it possible to distinguish between viruses of eastern or western origin by real time RT-PCR (Figure 3). Indeed, these assays provided the first indication that the outbreak of BT in northern Europe during August 2006 was caused by a western virus (data not shown). The potential problem of developing a single assay to detect Seg-1 from any of the viruses from either of these two distinct groups was overcome by combining two sets of topotype specific primers.

All 129 BTV isolates tested using the combined assay gave positive Ct results and amplification plots. These included field strains of BTV serotypes 1, 9, 16 (eastern genotype) and 1, 2, 4, 8, 11, 9 (western topotype) that were recovered from the European disease outbreaks since 1998. Only two strains of BTV gave inconsistent results, the South African BTV-9 vaccine (RSAvvv2/09) and a sample taken from a sheep just after vaccination with BTV-9 (ITL2003/01), which appears to be identical. In both cases dilution of the RNA test sample resulted in clear detection of the viral RNA, suggesting that assay may be susceptible to RT-PCR inhibitors present in the test sample that can be diluted out. However, these samples were extracted from a cell culture pellet and thus have extremely high concentrations of RNA. If the amount of RNA is excessive, then the number of binding sites for the primers and probes may overwhelm the concentrations of primer and probes in the reaction. This is unlikely to be an issue with diagnostic blood samples. Mismatches in the probe binding-site may also reduce the strength of probe-binding making it more sensitive to such inhibition. Variations in the concentration of inhibitors in the different preparations of RNA could explain why the other sample of the BTV-9 vaccine (RSAvvv1/09) gave consistently +ve results with the combined assay (despite having an identical Seg-1 target sequence). Such variations could therefore have a significant impact on the overall sensitivity and reliability of the assay. The nature and significance of the inhibitors needs further evaluation.

The ability of different BTV strains to reassort genome segments in the field has previously been demonstrated (Monaco et al., 2006a; Oberst et al., 1987; Sugiyama, 1981). The exchange of genome segments could potentially invalidate any diagnostic assays that can only detect one BTV genotype or isolates from a single geographic group, even if used to detect strains of a single serotype (a characteristic that is primarily controlled by genome segment 2 alone). The target footprints for a BTV specific rRT-PCR assay, should therefore be as highly conserved as possible across the whole BTV

virus species, to ensure that they can be used to detect any BTV strain, without cross-reacting with closely related viruses (like EHDV or AHSV).

BTV Seg-1 (the viral polymerase gene) is one of the most conserved regions of the virus genome (Huismans and Cloete, 1987; Mertens et al., 1987b), but has not previously been selected as a target for a BTV specific diagnostic RT-PCR assay, although since the assay described here was developed a second Seg-1 assay has been published (Toussaint et al., 2007b). The high level of sequence conservation in Seg-1 could potentially cause cross-reactions with closely related orbiviruses, as previously reported for Seg-3 (McColl and Gould, 1991). Indeed, it was possible to design Seg-1 specific primers that can be used to amplify Seg-1 from both EHDV and BTV (as used for sequence analyses). However, agarose gel electrophoresis showed that relevant cDNA bands were only generated in the rRT-PCR assay described here when it contained BTV RNA templates, indicating that amplification did not occur with the other closely related orbiviruses tested (Figure 3.5).

In situations where real-time RT-PCR machines are unavailable, the Seg-1 primer sets could be used in a conventional agarose gel based RT-PCR assay to detect BTV RNA. Although the amplicons are small, the conserved nature of Seg-1 suggests that by designing other primers, larger amplicons could be generated, which would be more suitable for electrophoretic analysis, whilst retaining specificity for the BTV serogroup. Since EHDV is recognised as the *Orbivirus* species most closely related to BTV (Mertens, 2005), its failure to amplify suggests that the assay will be specific for members of the BTV species.

BTV RNA was detected successfully in homogenates of *C. sonorensis*, (7-10 days after infection by membrane-feeding with BTV-9 infected blood) using the combined pan-BTV specific assay (Table 2). Individuals or pools of field caught midges could therefore be tested for the presence of virus using this assay, potentially providing information on virus transmission during an outbreak (Veronesi et al., 2008). Although this approach has been used during the current European outbreak of BTV-8, (Mehlhorn et al., 2007; Vanbinst et al., 2009), it is important to remember that detection of viral RNA does not necessarily equate to infectious virus (Carpenter et al., 2008a).

The combined Seg-1 rRT-PCR assay could be used to detect less than ten template copies per reaction. From previous analyses of purified BTV particle infectivity in cell culture, this is equivalent to approximately 0.5 TCID₅₀/ml for disaggregated virus particles in BHK-21 cells (Mertens et al., 1996). This indicates that the assay is

approaching the theoretical sensitivity limits of such a system and is significantly more sensitive than the simple detection of virus by isolation in cell culture.

Multiple assays with uninfected blood or insect samples, containing ovine, bovine or *Culicoides* nucleic acids, consistently failed to 'amplify', demonstrating that cross-reactions with host sequences would not generate false positive results.

Three out of six samples of sheep blood originally sent to the CRL for testing, from the Netherlands at the start of the European Outbreak of BTV-8, were positive in the combined probe assay. Two of these samples were also confirmed as BTV +ve by cELISA to detect BTV specific antibodies (Anderson, 1984) and all three were +ve by a conventional Seg-7 specific RT-PCR (Anthony et al., 2007). Six samples of cattle blood taken during the BTV outbreaks in Spain during 2005 were also tested with the dual-probe rRT-PCR assay. Five of the six samples were positive for virus isolation, resulting in virus isolates SPA2005/01 to SPA2005/05. However, all six blood samples gave clear positive results when tested using the dual-probe rRT-PCR assay.

Clinical samples from remote areas sometimes arrive at the IAH BTV reference laboratory in far from ideal condition, resulting in partial or complete degradation of virus particles and a subsequent loss of infectivity, making virus isolation difficult or impossible. However, the combined Seg-1 assay can be used to detect viral RNA in blood or tissue samples, even where the virus itself is degraded or non-infectious. Indeed viral RNA was detected successfully in sonicated RSAvvvv/16 infected ovine blood samples that had been stored for over a year at +4 °C (data not shown). The assay could also be used to detect non tissue-culture adapted viruses, and non-infectious virus in the post-viraemic stage of infection (MacLachlan et al., 1994) and it is certainly more rapid than virus isolation, which can take several weeks.

One unexpected outcome of the study is the development of separate assays (containing only a single primer set) that could be used to detect and distinguish Seg-1 from eastern or western BTV topotypes. When combined in a single assay these primers could be used to detect any of the BTV isolates tested regardless of topotype.

The combined assay represents a major improvement in the standard diagnostic techniques used by the CRL to detect BTV. It now forms part of routine testing procedures for samples of blood, tissue and virus isolates by the CRL (and several other reference labs across Europe) and has proved to be rapid, sensitive and BTV-specific, with over 21,000 clinical samples tested to date.

The assay was found to work well when it was used in a quantitative or semi-quantitative manner. Quantitative RT-PCR analysis of cell cultures infected with RSAvvv/16 revealed an initial delay in replication, followed by a phase of rapid increase before a sustained period where, although the rate of change was reduced, the speed continued to increase. These data concur with previous work which has demonstrated virus release as early as four hours post infection (Hyatt et al., 1989). After the initial 'lag phase' for entry, uncoating and activation of the core, it appears that there is a period when BTV RNA accumulates rapidly through transcription from core particles and the formation of progeny virions.

The outbreak of BTV-8 in Northern Europe demonstrated a need for a robust and high-throughput real-time RT-PCR assay. The most obvious way in which to increase the throughput of diagnostic assays is to use robotics. Robotic apparatus can extract nucleic acid and 'set-up' RT-PCR reactions. The chemical denaturation of viral dsRNA is highly efficient but, uses a highly toxic chemical (methyl mercuric hydroxide) and reaction volumes that are below the minimum required for accurate pipetting by robots. Heat denaturation of viral dsRNA in test samples allows the use of larger pipetting volumes, within the range used by robotic systems, and has no negative effect on assay sensitivity or efficiency. Automated nucleic acid extraction now represents the standard protocol used in the CRL at IAH Pirbright; increasing throughput and reducing the steps where there is potential for human error and contamination.

Although the original assay, containing two probes appeared robust, there was concern that the overlapping probe footprints could interfere with detection of challenging samples, as observed during analysis of a highly concentrated RNA sample of the BTV-9 vaccine - RSAvvv2/09. A single probe containing two redundancies was tested in both the sense and antisense orientations. Initial experiment showed that although the 'sense' probe had similar characteristics to the dual probe assay, the antisense version of the probe orientation had improved amplification characteristics, including steeper amplification curves and a higher plateau of fluorescence. It is not clear how the different probe-orientation influences the level of fluorescence, although it may be related to the number and position of guanosine bases (which possess quenching properties, (Cooper and Hagerman, 1990). Further testing demonstrated that the antisense probe was also less susceptible to factors in the RSAvvv2/09 RNA-sample which had inhibitory effects on the original assay. It is considered that differences in the binding characteristics of this probe, may affect the susceptibility of the assay to 'inhibition'.

Molecular diagnostic assays are being commercialised by several companies, including the VetMax™ range from Applied Biosystems, the TaqVet® range from Laboratoire Service International, and the *cador* range from Qiagen. The original 'dual probe' assay has now been commercialised by Applied Biosystems.

The IAH has entered into a commercial agreement with Qiagen to commercialise the Seg-1 rRT-PCR assay, incorporating the RedunR probe. The initial testing of the prototype kit highlighted an inability to reliably detect BTV in blood samples that were found weak positive using the Shaw *et al* V2.0. Competition for reaction components was ruled out by using the assay with a dilution series of the IPC, as well as testing samples with and without the IPC. However, denaturation of RNA samples in an identical fashion to the original dual-probe assay, resulted in reproducible detection of samples, including weak positive blood samples containing BTV-11, which had tested negative in the absence of heat denaturation.

The requirement for heat denaturation of weak samples implies that the template is double-stranded particularly in these weaker samples. the presence of RNA samples which are both low concentration and primarily double-stranded suggests that the infection is advanced to a point where the virus is replicating slowly, or not at all and therefore producing little (if any) single stranded mRNA (Brewer and MacLachlan, 1994b; Stott *et al.*, 1990). This contrasts to an early infection, which would also give a weak signal, but where ssRNA would be abundant in the form of mRNA. It may therefore be possible to use the assay with or without denaturation, to distinguish early and late infections in the host. However this will require further work and evaluation.

More extensive testing revealed that the Qiagen assay kit maintained the ability of pan-serotypic detection of while maintaining specificity for the BTV serogroup. The only samples which caused detection problems for the kit were the same ones that caused problems during the initial development of the assay. Dilution of the concentrated sample solved the issue, again suggesting some form of concentration dependent block or inhibition. The fact that the IPC was only amplified at a dilution of 1:2000 provides more evidence that an inhibitory factor may be causing the lack of amplification observed with these samples.

The 'clinical sensitivity' of the assay for a selection of RNAs which had been extracted from blood samples using Qiagen spin-column technology was 90%. One of the samples was only detected when 5 µl of RNA was used instead of 10 again suggesting

inhibition of the PCR reaction. However, the IPC was still positive demonstrating that the amplification conditions were effective..

This chapter outlines and discusses the development, evaluation and subsequent commercialisation of a pan-BTV real-time RT-PCR assay. This assay has been adopted by the CRL for BTV at Pirbright as well as numerous other laboratories around the world as a frontline diagnostic assay. The diagnostic impact of this assay will inevitably be increased by its availability as a commercial kit from Qiagen. In addition to its diagnostic use, the assay is valuable as a research tool for the quantitative or semi-quantitative detection of BTV-RNA.

Chapter 4: Cloning the genes of the BTV non-structural proteins and their transient expression in mammalian cells

4.1: Introduction

Bluetongue virus can cause a near 100 % shut-off of host cell protein synthesis during infection of mammalian cells (Huismans, 1970b). Although the precise mechanism of shut-off has not been fully elucidated for any of the reoviruses, it has been reported that orthoreovirus switches from a cap dependent to a cap-independent method of protein synthesis, and consequently the uncapped mRNAs derived from progeny virus particles are translated with greater efficiency (Skup and Millward, 1980). A previous study has also indicated that BTV may initiate a similar transition from cap-dependent to cap-independent translation in mammalian cells, but not in insect cells (Stirling, 1996). Indeed, a lack of host-cell protein synthesis inhibition is a characteristic of BTV replication in insect cells and is thought to be a necessary factor in establishing their persistent infection.

A persistent state of infection in mammalian cells was previously reported after formation of BTV-NS1 tubules was disrupted by intracellular expression of a single chain NS1 specific antibody fragment (scFv). This allowed the infected cells to behave in a fashion similar to insect cells persistently infected with BTV (Owens et al., 2004). The authors proposed that the relative levels of NS3/3A to NS1 expression, determines the level of cytopathology caused in mammalian cells by BTV infection.

Over-expressing NS1 or NS3/3A would therefore be predicted to alter the outcome of infection, for example if NS3/3A was over expressed in mammalian cells, a persistent infection (instead of the severe lytic infection) would result. Similarly, reducing the level of NS1 in mammalian cells would be expected to result in a persistent infection.

The effect of the relative expression levels of NS1 or NS3/3A, on BTV replication and infection, was examined in BHK-21 cells. The approach chosen, was to clone the relevant viral gene into an expression vector under the control of the strong cytomegalovirus (CMV) promoter. This could then be used to 'over-express' the BTV protein in the host cell.

Efforts during this project to generate antibodies in rabbits and guinea pigs, against peptides derived from BTV NS3 were unsuccessful and no antibodies were available to detect native NS3/3A protein. In addition, very little antibody was available for the detection of NS1. The BTV genes were therefore cloned with epitope tags (V5

and 6HIS) or enhanced green fluorescent protein (EGFP). Plasmid vectors are commercially available, which have multiple cloning sites for insertion of viral genes, simplifying the over-expression of BTV proteins in mammalian cells, using the CMV promoter. Once sequenced to confirm the authenticity of the cDNA insert, these plasmids can be used in transfection experiments to determine whether there is an observable difference in the outcome of infection as a result ofk NS1 or NS3/3A over expression (i.e. persistence vs. lysis).

It has been suggested that high levels of NS1 synthesis in BTV infected mammalian cells (relative to the levels of NS3/3A), results in a lytic infection (Owens et al 2004). This hypothesis could be tested by reducing the relative level of NS1, or increasing the levels of NS3/3A expression.

One possible way of reducing expression of a specific gene is to induce post transcriptional gene silencing (RNA silencing). Small interfering dsRNAs (20 - 25 bp - siRNAs) could be produced from full-length viral dsRNAs by treatment with Dicer (RNase III endoribonuclease) (Aalto et al., 2007). One strand of the siRNA (the guide strand) becomes incorporated into an RNA-induced silencing complex (RISC), which would then be capable of cleaving ssRNAs with a sequence matching the siRNA strand. Silencing of specific BTV genes could be induced by transfection of these siRNAs into infected cells. The RNA silencing pathway has previously been used for both therapeutic and research purposes, by delivering pre-made siRNAs into cells.

Different methods exist for the production of siRNAs. A common approach for artificial production of siRNAs is to chemically synthesise the desired siRNA. An alternative method of siRNA production is to digest *in vitro* a stretch of dsRNA representing the target gene using recombinant Dicer (H. Attoui, personal communication). This project used the latter approach as it was anticipated that the greater diversity of siRNAs generated representing the entire length of Seg-5 would result in a more efficient RNA silencing response.

4.2: Shut-off of host cell protein synthesis by BTV

The levels of host-cell protein synthesis and the timing at which host-cell shut-off occurs, were analysed by ‘pulse-stop’ metabolic labelling with [³⁵S]-methionine / cysteine, over a time-course post BTV infection (Figure 4.1).

Decreasing amounts of radiolabel were incorporated into host cell proteins after approximately eight hours post BTV infection (Figure 1). The only exception was one

host cell protein which is thought to be actin (Huismans, 1970b), demonstrating an almost complete shut-off of host cell protein synthesis. Bands representing the individual BTV proteins also became visible by eight hours, and by 16 hours post infection the only proteins being made were viral in origin (Figure 4.1).



Figure 4. 1: Metabolic labelling of BHK cells infected with BTV.

BHK-21 cells seeded into 35 mm dishes were incubated for 24 hours to reach 95% confluence. BHK cell sheets were rinsed with serum free media, which was subsequently replaced with 1 ml of a 10 % (v/v) dilution of RSAvvvv/16 cell culture material in GMEM (or plain GMEM for mock infections), resulting in an MOI of approximately 10. The cells were incubated at room temperature for 1 hour at 37 °C before adding an additional 1 ml of media. At selected time points post infection the cells were pulse-labelled with [³⁵S] Met/Cys for two hours without prior starvation. For pulse-labelling, the media was replaced by 2.5 ml of methionine / cysteine deficient Eagle's medium containing 0.3 MBq of [³⁵S] labelled methionine / cysteine Promix. The cells were incubated at 37 °C for two hours before 'safely' discarding the media and harvesting the cells using 250 µl of 2 × SDS sample buffer containing DTT. The sample lysates were separated using SDS-PAGE and subjected to autoradiography (Materials and Methods, section 2.11.2). Viral proteins become increasingly abundant from approximately eight hours post infection with a concurrent decrease in host cell labelling. By 16 hours post infection the proteins synthesised are primarily viral.

4.3: Cloning Segment-10 (NS3/3A)

4.3.1: pcDNA3.1 V5/HIS

In order to construct a plasmid for over-expression of BTV NS3/3A in mammalian cells, BTV Seg-10 RNA was reverse-transcribed from a purified viral RNA using MMLV, amplified by PCR, and cloned into pcDNA3.1 V5/HIS (described in Material and Methods, section 2.16.2). The RSAvvvv/16 segment-10 sequence was obtained by sequencing the remaining PCR product. The resulting plasmid, pcDNA3.1 V5/HIS_NS3/3A, was also sequenced to ensure the authenticity of the construct (Figure 4.2).

This plasmid expressed both NS3 and the shorter form, NS3A. It is therefore not possible to differentiate any localisation differences between the proteins. Mutation of either of the start codons would have allowed specific expression of each form independently, although the methionine at amino acid position 14 would be mutated in the resulting NS3 protein. Four of the first six amino acids of NS3 are hydrophobic, perhaps reflecting the membrane associated nature of the NS3 protein. The stretch of amino acids between 8 – 23 contains 69% charged residues (5 positive, 6 negative amino acids). The other domains reported in NS3/3A were also observed, namely the proline-rich late domain, two transmembrane regions and a N-linked glycosylation site in the extracellular region (Figure 4.2).

RSAvvvv/16 NS3/3A

Start of insert = nt 1

nt 1-6 GCTAGC = NheI restriction site

Start codons (ATG) = nt 26-28 [NS3] + 65-67 [NS3A]

Potential late domain region (PPRYAPSAP) = amino acids 36-44 (Wirblich et al. 2006)

Transmembrane domain I = amino acids 118–141 (Bansal et al., 1998).

Transmembrane domain I = amino acids 118-141 (Bansal et al., 1998); Transmembrane domain II = amino acids 162-182 (Bansal et al., 1998).

Region between transmembrane domains = amino acids 142-161

Region between transmembrane domains = amino acids 142-161
 Plasmid-derived epitope tags (multiple cloning site) = nt 714-747

Plasmid derived epitope

Figure 4.2: The sequence of the RSA_{vvvv}/16 NS3/3A gene

The sequence of the clone pcDNA3.1 V5/HIS_NS3/3A was identical to the sequence obtained by sequencing the left over PCR product from the cloning procedure. The published features of NS3/3A were all present and are highlighted. The NheI and SacII restriction sites were used for subsequent cloning steps. nt = nucleotide positions

4.3.2: pEGFP-NS3/3A and pNS3-EGFP

NS3/3A was fused to a fluorescent molecule, (enhanced green fluorescent protein (EGFP)) for transient expression and live cell imaging. The ligation was transformed into DH5 α cells and produced abundant colonies. Five colonies were analysed by restriction digest using NheI and SacII, three of which gave a band of the correct size for NS3/3A (Figure 4.3). These positive clones were sequenced using primers listed in Table 2.6, to confirm that the insert was correct. A maxiprep was made for clone ‘pNS3/3A-EGFP-cl1’.

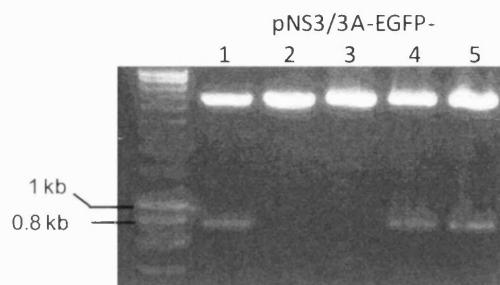


Figure 4. 3: Restriction digest analysis of pNS3/3A-EGFP colonies.

A SacII/NheI insert excised from pcDNA3.1V5/HIS_NS3/3A ligated into pEGFP-N1 (Figure 4.4) digested with the same enzymes. The ligation was transformed into DH5 α competent cells. Five kanamycin-resistant colonies were amplified in LB broth and purified by miniprep. 3 μ l of the miniprep was digested for 2 hours using SacII and NheI and analysed by AGE. Clones 1, 4 and 5 contained an insert of the expected size and were sequenced.

Previous reports indicate that both termini of NS3 perform specific functions involved in the release of progeny BTV particles from infected cells (Beaton et al., 2002). As both termini of the protein are thought to be important, a second EGFP construct was generated with EGFP fused to the N terminus of NS3, as opposed to the C terminus.

Abundant colonies were obtained with five selected for further analysis by miniprep and restriction digest. A kanamycin resistant clone was selected and, upon confirmation of an insert by restriction digest, sequenced using the primers in Table 2.6. The correct sequence was confirmed prior to amplification by ‘maxiprep’.

4.3: Transient expression of NS3

No anti NS3/3A specific antibodies were available during this project. The intracellular distribution of NS3/3A was therefore compared to previous observations by (Bansal et al., 1998).

BHK-21 cells were seeded onto 13 mm diameter coverslips in 24 well plates. After 24 hours they were transfected with pcDNA3.1V5/HIS_NS3/3A. Then at 24 hours

post-transfection the cells were fixed using 4 % paraformaldehyde and immunolabelled using an α V5 mAb and a Golgi-specific antibody (GM130, Table 2.12, Chapter 2, Materials and Methods).

The distribution of NS3/3A-V5 matched published findings for NS3/NS3A (Bansal et al., 1998; Hyatt et al., 1991). The protein was primarily localised to either the plasma membrane, (as revealed by DIC imaging), or an intracellular agglomeration that co-localised with the Golgi apparatus (Figure 4.4). There were also abundant cytoplasmic dots which may represent the smooth surfaced vesicles which become abundant in BTV infected cells. These features match components of the secretory pathway, with which BTV NS3/3A has been linked (Wirblich et al., 2006).

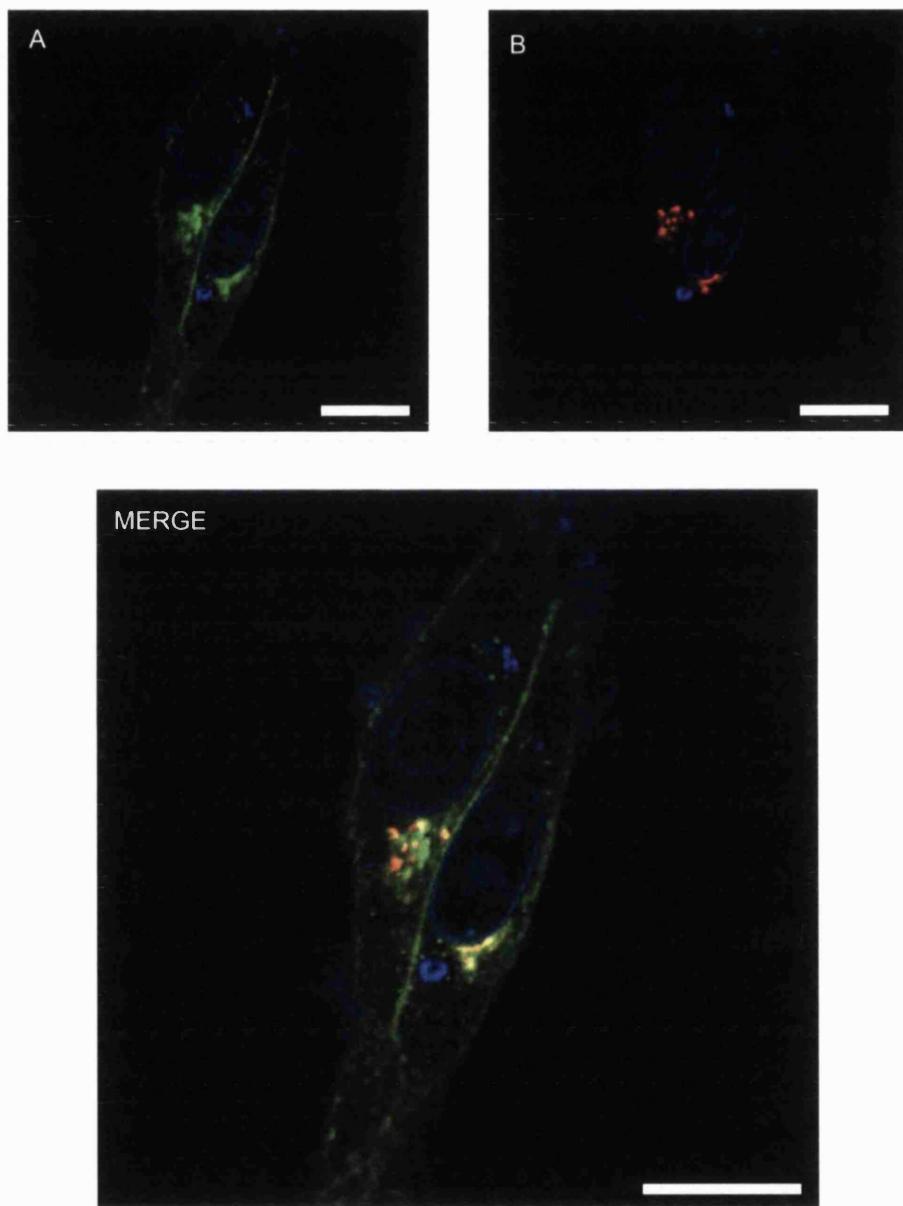


Figure 4. 4: The expression and localisation of NS3/3A-V5

BHK-21 cells were seeded on glass coverslips and incubated for 24 hours. The cells were transfected with pcDNA3.1V5/HIS_NS3/3A. The cells were fixed using 4% paraformaldehyde at 24 hours post transfection and immunolabelled using antibodies to the V5 epitope and GM130 (Table 2.12, Chapter 2, Materials and Methods). Nuclei were stained using DAPI (blue). Expressed NS3/3A-V5 (green) demonstrated the expected intracellular and plasma membrane distributions (A). The Golgi-specific labelling using GM130 (red, B) localised to a perinuclear position. Anti-V5 labelling co-localised with the Golgi (Merge).

Scale bar = 10 μ m

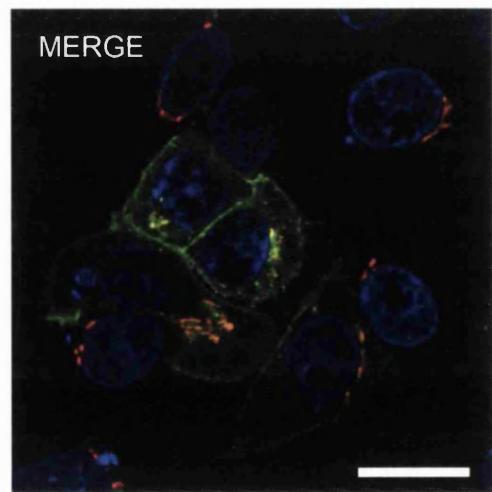
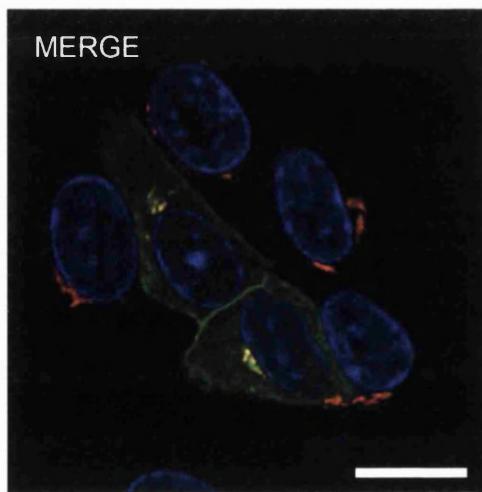
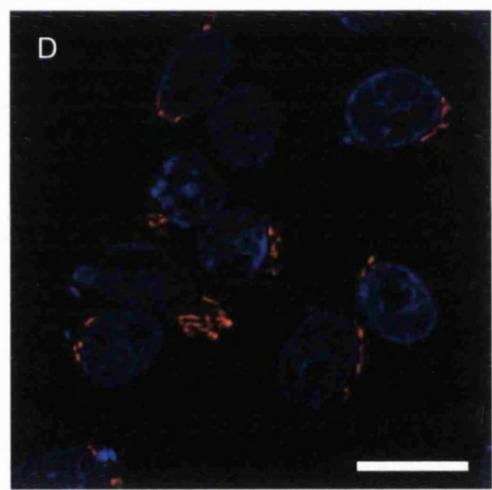
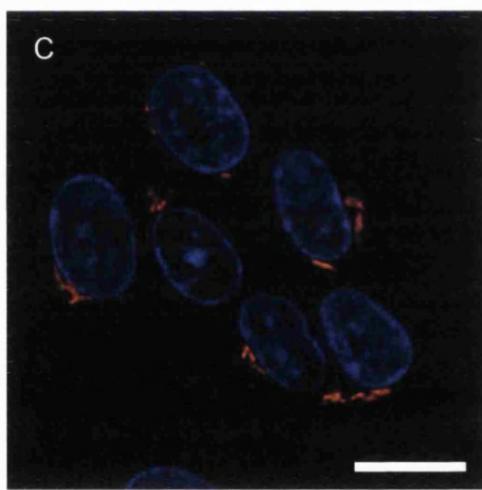
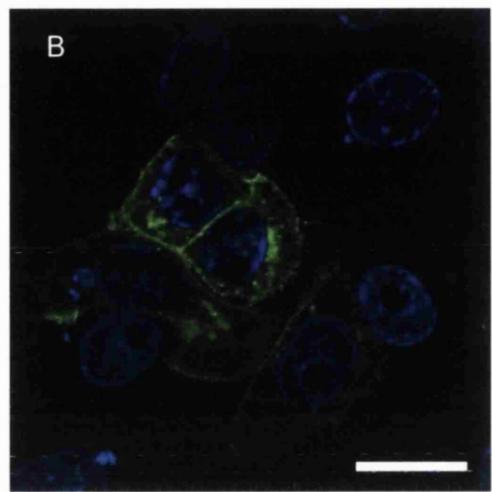
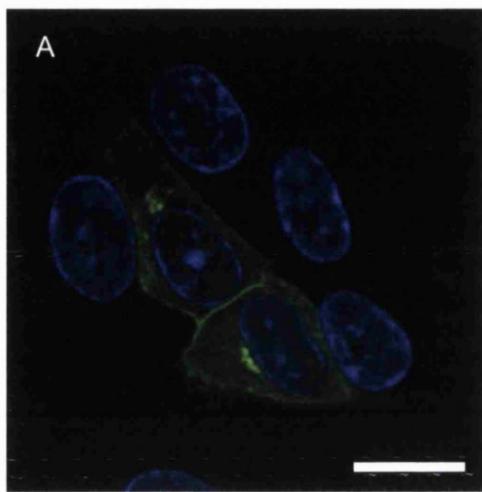
The distribution of NS3/3A-EGFP and EGFP-NS3/3A was also analysed using confocal microscopy in a manner similar to pcDNA3.1V5/HIS_NS3/3A. BHK-21 cells which had been seeded onto coverslips were transfected with pNS3/3A-EGFP or pEGFP-NS3/3A and fixed at 24 hours post transfection using 4 % paraformaldehyde. The cells were immunolabelled with GM130 (Table 2.12, Chapter 2, Materials and Methods). Both NS3/3A-EGFP and EGFP-NS3/3A signal co-localised with the Golgi labelling in a similar manner to NS3/3A-V5 (Figure 4.5).

[Figure next page]

Figure 4. 5: The expression and localisation of EGFP tagged NS3/3A.

BHK-21 cells were seeded on glass coverslips and incubated for 24 hours. The cells were transfected with pNS3/3A-EGFP (A, C and Merge) or pEGFP-NS3/3A (B, D and Merge). The cells were fixed using 4 % paraformaldehyde at 24 hours post transfection and immunolabelled using the Golgi-specific antibody GM130 (Table 2.10, Chapter 2, Materials and Methods). Nuclei were stained using DAPI (blue). Expressed NS3/3A fused to EGFP (A and B, green) was found to be distributed in an identical pattern as that observed for NS3/3A-V5, i.e. localising to the Golgi (B and D, red) and the plasma membrane.

Scale bar = 10 μ m

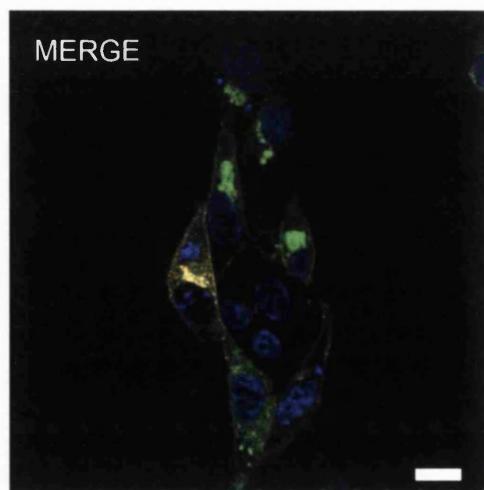
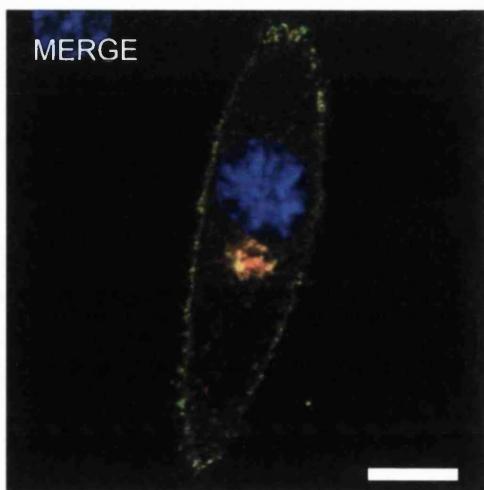
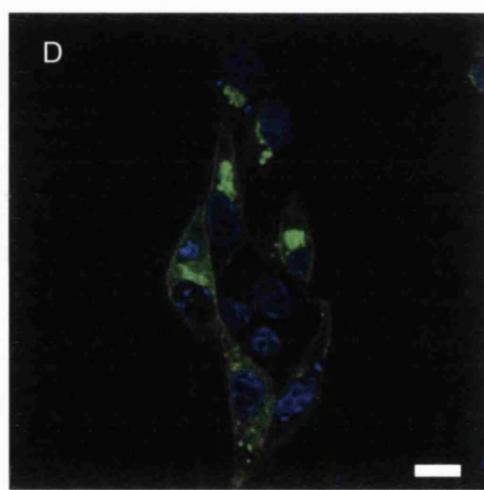
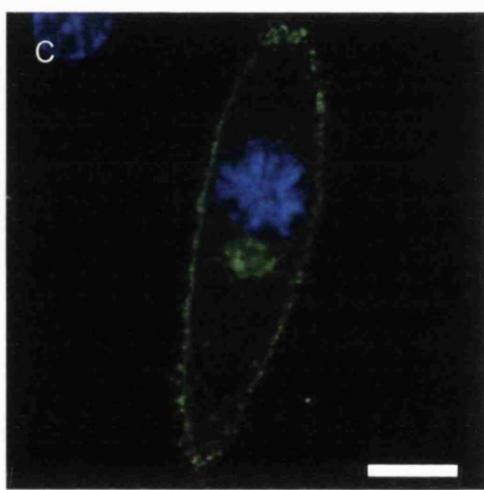
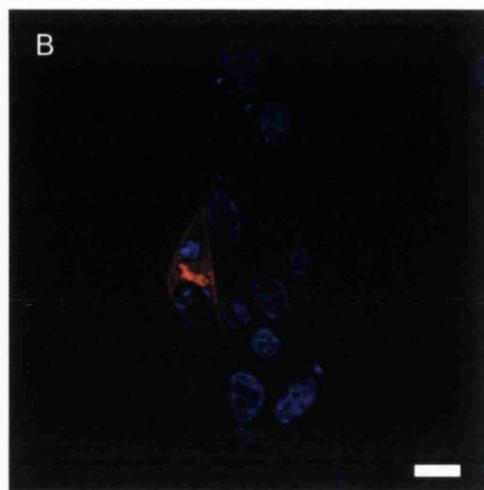
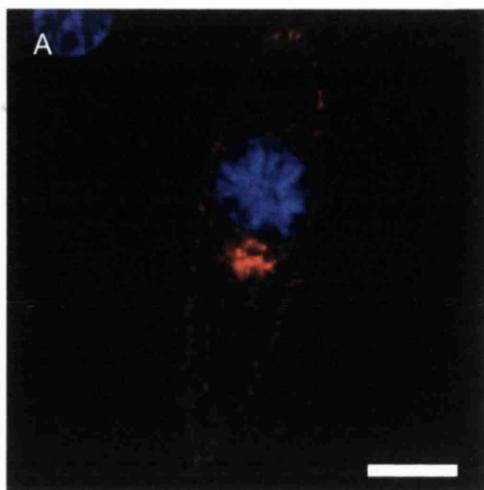


The impact of the fused EGFP to the native protein on the distribution of NS3/3A was also assessed using confocal microscopy. Transfection procedures identical to those above were performed using transfection complexes containing pcDNA3.1V5/HIS_NS3/3A and either NS3/3A-EGFP or EGFP-NS3/3A and, after fixation immunolabelling for the V5 epitope expressed on NS3/3A-V5. It was found that the NS3/3A-V5 and the NS3/3A-EGFP or EGFP-NS3/3A co-localised together (Figure 4.6).

No fluorescent protein (NS3/3A-EGFP or EGFP-NS3/3A) was observed in the nucleus. This distribution is also different from native EGFP, which is uniformly distributed throughout the cytoplasm and nucleus. Together, these data indicate that the NS3/3A protein sequence dictates the localisation of the fusion protein within the cell, which was not affected by the presence of EGFP at either terminus.

[Figure next page]

Figure 4. 6: The co-localisation of expressed NS3/3A-V5 with EGFP tagged NS3/3A. BHK-21 cells were seeded on glass coverslips and incubated for 24 hours. The cells were cotransfected with pcDNA3.1V5/HIS_NS3/3A and either pNS3/3A-EGFP (A, C and Merge) or pEGFP-NS3/3A (B, D and Merge). The cells were fixed using 4 % paraformaldehyde at 24 hours post transfection and immunolabelled using antibodies to the V5 epitope (Table 2.10, Chapter 2, Materials and Methods). Nuclei were stained using DAPI (blue). Expressed NS3/3A-V5 (A and B, red) was found to be co-localise to the Golgi and plasma membrane with NS3/3A with EGFP (B and C, green) fused to either the carboxy terminus (A, C and Merge) or amino terminus (B, D and Merge). Scale bar = 10 μ m



4.4: Live cell imaging of NS3/3A

Live cell imaging was used to assess whether NS3/3A-EGFP, or EGFP-NS3/3A, were trafficking to the plasma membrane of the cell, in a manner that would support the hypothesis that NS3/3A is responsible for virus egress (Beaton et al., 2002).

BHK-21 cells were seeded into glass bottom 35 mm dishes (Bibby Sterilin) and transfected 24 hours later with pEGFP-NS3/3A or pNS3/3A-EGFP. After 16 hours to allow expression, cells expressing the fluorescent protein were selected for live cell imaging with a Leica DMIRE2 inverted confocal microscope with incubated housing. Cells were imaged for 30 minutes to track the movement of the EGFP. The protein was located at the plasma membrane and Golgi apparatus in a pattern similar to that previously observed in fixed cells. However, very little movement in the protein's distribution was observed during the imaging period (data not shown). The results were the same regardless of the infection status of the cell. Both of the NS3/3A fusion constructs were tested in live cell imaging with identical results.

4.5: Segment-5 (NS1)

4.5.1: pcDNA3.1 V5/HIS

A similar strategy was used to assemble a plasmid for expression of epitope tagged NS1, using topoisomerase T-A cloning into pcDNA3.1 V5/HIS, to generate pcDNA3.1 V5/HIS_NS3/3A (Materials and Methods, section 2.16.3).

However, less than 30 colonies were produced. All of the colonies were selected for further analysis and miniprep cultures in LB broth. Purified plasmid DNA (Materials and Methods, section 2.19.3) was analysed by restriction digestion with BstXI (for which there are sites either side of the insert), only a single clone (clone 27) contained an insert of the appropriate size for a full length copy of the NS1 gene (Figure 4.7). Typically either the insert was not present, or large sections of the BTV Seg-5 sequence were deleted.

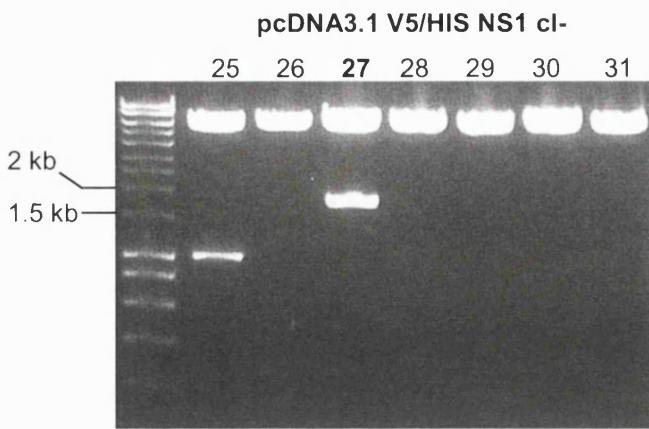


Figure 4. 7: Restriction digest analysis of pcDNA3.1V5/HIS NS1.

A PCR product encompassing the BTV-16v NS1 open reading frame (minus stop codon) was inserted into the pcDNA3.1V5/HIS cloning kit (Invitrogen). The colonies evident after transformation of the cloning reaction into competent TOP10 bacteria were picked and grown in LB broth and used for plasmid purification using the miniprep kit (Qiagen). 3 μ l of the purified plasmid was subjected to restriction digest analysis using BstXI and separated using AGE. Only ‘clone 27’ contained a full-length insert (1690 bp).

Comparisons of the sequence of PCR products from Clone 27, showed that it contains two nucleotide differences from the consensus sequence, one of which was ‘non-synonymous’ (amino acid 89, phenylalanine to serine, [F89S], Figure 4.8). Although the loss of the phenylalanine results in a slight reduction in hydrophobicity, this mutation has potential significance. Serine residues are often phosphorylated in order to modify the function of a protein and this mutation results in a series of three serines. The BTV NS1 protein appears to be highly structural and therefore such internal bases may be of lesser importance. Nevertheless, in the absence of a firmer understanding of the role of NS1 this mutation must be taken into consideration. However, based upon the difficulty of obtaining even a single clone, it was decided that this mutation was acceptable.

RSAvvvv/16 NS1

1	21	41	61	81
GTAAAGGTTCTAGTGGCAACCACCAAC	ATGAGCGCTTTGAGAAAATACAACATCAGTGGGATTATGCTAATGC			
<u>GCTAGCGT</u> AAAAAGTTCTAGTGGCAACCACCAAC	ATGAGCGCTTTGAGAAAATACAACATCAGTGGGATTATGCTAATGC			
	M E R F L R K Y N I S G D Y A N A			
101	121	141	161	
CACGAGAACTTTGGCTATTCACCTCAATGGACTTGCAGTCAC	TTAAAGGAATTGTTATTTAATGGATGTGTCAAACAAAA			
CACGAGAACTTTGGCTATTCACCTCAATGGACTTGCAGTCAC	TTAAAGGAATTGTTATTTAATGGATGTGTCAAACAAAA			
T R T F L A I S P Q W T C S H L K R N C L F N G M C V K Q N				
181	201	221	241	261
TTTGAAAGAGCAATGATTGAGCAACTGATGCTGAAGAACCA	CACGGAAAGCATTCAACTGGTTGAAATTAGCTAAAGAGGCCATGT			
TTTGAAAGAGCAATGATTGAGCAACTGATGCTGAAGAACCA	CACGGAAAGCATTCAACTGGTTGAAATTAGCTAAAGAGGCCATGT			
F E R A M I A A T D A E E P R K A F Q L V	E L A K E A M Y D			
281	301	321	341	

| | | | |
 TCGAGAGACGGTTGGCTCAAATGTTCAAAAGTTCTCCAACCGTATGAAGAGGACATCGAAGGAAAGATGAGGAGGTGGCGTACA
 TCGAGAGACGGTTGGCTCAAATGTTCAAAAGTTCTCCAACCGTATGAAGAGGACATCGAAGGAAAGATGAGGAGGTGGCGTACA
 R E T V W L Q C F K S P S Q P Y E E D I E G K M R R C G V Q


 361 381 401 421 441
 | | | | |
 GCTGCTCGAAGATTACCGCAAAAGTGGATGATGGATGAGCGGTGAAGCAGTCGCATTGAACAAATTCAAGAGGAGTAGATTGGATGA
 GCTGCTCGAAGATTACCGCAAAAGTGGATGATGGATGAGCGGTGAAGCAGTCGCATTGAACAAATTCAAGAGGAGTAGATTGGATGA
 L L E D Y R K S G M M D E A V K Q S A L N N S E R I R L D D

 461 481 501 521
 | | | | |
 TCGCTGTCAGCAATGCCATACATCTATGACCAATCAGAGAAGGCCAATCGTTAATCCAACATTCTCGAGATATCGTCAAATCGC
 TCGCTGTCAGCAATGCCATACATCTATGACCAATCAGAGAAGGCCAATCGTTAATCCAACATTCTCGAGATATCGTCAAATCGC
 S L S A M P Y I Y V P I R E G Q I V N P T F I S R Y R Q I A

 541 561 581 601 621
 | | | | |
 ATATTATTTTATAATCCTGATGCTGCAGATGATTGGATCGATCCAATTGTTCGGAATCCGAGGGCAACACAATCAGATAAAACGGGA
 ATATTATTTTATAATCCTGATGCTGCAGATGATTGGATCGATCCAATTGTTCGGAATCCGAGGGCAACACAATCAGATAAAACGGGA
 Y Y F Y N P D A A D D W I D P N L F G I R G Q H N Q I K R E

 641 661 681 701
 | | | | |
 AGTAGAGAGGCAAAATTAAATACATGTCCTTATTCAAGGCTACAAAGGTAGGGCTTTCAAGTGTGATTTTACCAATCCAAATGATAAAATT
 AGTAGAGAGGCAAAATTAAATACATGTCCTTATTCAAGGCTACAAAGGTAGGGCTTTCAAGTGTGATTTTACCAATCCAAATGATAAAATT
 V E R Q I N T C P Y S G Y K G R V F Q V M F L P I Q M I N F

 721 741 761 781 801
 | | | | |
 TCTGAGAATGGATGATTTCGCGAAGCACTTAAATAGATATGCTCGATGGCTATCCAACAGTATCTGAGAGTCGGTATGCTGAAGAAGT
 TCTGAGAATGGATGATTTCGCGAAGCACTTAAATAGATATGCTCGATGGCTATCCAACAGTATCTGAGAGTCGGTATGCTGAAGAAGT
 L R M D D F A K H F N R Y A S M A I Q Q Y L R V G Y A E E V

 821 841 861 881
 | | | | |
 CAGATATGTCAACAACTGTTGGAAAGTCCAACCTGGTAATTCCATTGCACTCAGATGATGTTGAGACCGCAGTCCCCACGCG
 CAGATATGTCAACAACTGTTGGAAAGTCCAACCTGGTAATTCCATTGCACTCAGATGATGTTGAGACCGCAGTCCCCACGCG
 R Y V Q Q L F G K V P T G E F P L H Q M M L M R R D F P T R

 901 921 941 961 981
 | | | | |
 CGACCGCAGCATTGTTGGAATCACCGCTGAGGAGATCGGGTGTGAAAGCTGGCAACAGCTGGTTATTACCAATGATTAGTGGGGAAAGG
 CGACCGCAGCATTGTTGGAATCACCGCTGAGGAGATCGGGTGTGAAAGCTGGCAACAGCTGGTTATTACCAATGATTAGTGGGGAAAGG
 D R S I V E S R V R R S G D E N W Q S W L L P M I I V R E G

 1001 1021 1041 1061
 | | | | |
 ATTGGACCAACCGGAGAGATGGAGTGCTCTAGATTATGGACAGAAAGCATACTGTCAACTATGCTATCTAAACATTCAAAGCA
 ATTGGACCAACCGGAGAGATGGAGTGCTCTAGATTATGGACAGAAAGCATACTGTCAACTATGCTATCTAAACATTCAAAGCA
 L D Q P E R W E W L L D Y M D R K H T C Q L C Y L K H S K Q

 1081 1101 1121 1141 1161
 | | | | |
 GATACAGACATGTGGTGTGATGATGTGCGTCATCGAAATTGATGGGGTGTACCTTCAGAACGGTTAAGATCGAAGAGCATGTGGG
 GATACAGACATGTGGTGTGATGATGTGCGTCATCGAAATTGATGGGGTGTACCTTCAGAACGGTTAAGATCGAAGAGCATGTGGG
 I Q T C G V I D V R A S E L I G C S P F R T V K I E E H V G

 1181 1201 1221 1241
 | | | | |
 TAATGATTCAAGACAAAGCTGGTCGTGATGAGCAGATTGGCGGATTGGGGATCATACTATACAAACAAATTGTTACACAGG
 TAATGATTCAAGACAAAGCTGGTCGTGATGAGCAGATTGGCGGATTGGGGATCATACTATACAAACAAATTGTTACACAGG
 N D S V F K T K L V R D E Q I G R I G D H Y Y T T N C Y T G

 1261 1281 1301 1321 1341
 | | | | |
 CGCTGAAGCATGATCACGACAGCCATTACACATACATCGATGGATAAGAGGGTGTGGCATCTGGATGAGAAGGGATGGCAGGAAGGTAT
 CGCTGAAGCATGATCACGACAGCCATTACACATACATCGATGGATAAGAGGGTGTGGCATCTGGATGAGAAGGGATGGCAGGAAGGTAT
 A E A L I T T A I H I H R W I R G C G I W N D E G W Q E G I

 1361 1381 1401 1421
 | | | | |
 CTTCATGTTGGGCGTGTGTTGAGGGGGAGTTAACGAAGGCGCAACGCGAGCGCGCTGCTTAGACTCTCTGCTTCGTATGTTACGG
 CTTCATGTTGGGCGTGTGTTGAGGGGGAGTTAACGAAGGCGCAACGCGAGCGCGCTGCTTAGACTCTCTGCTTCGTATGTTACGG
 F M L G R V L L R W E L T K A Q R S A L L R L F C V C Y G

1441	1461	1481	1501	1521
ATATGCGCCACCGCGCAGATGGAACAAATGCCGGACTGGAATAACTTGGTAGTTCTTGACATTATACTGAAGGGACCAGAACCTGGTGA				
ATATGCGCCACCGCGCAGATGGAACAAATGCCGGACTGGAATAACTTGGTAGTTCTTGACATTATACTGAAGGGACCAGAACCTGGTGA				
Y A P R A D G T M P D W N N L G S F L D I I L K G P E L G E				
1541	1561	1581	1601	
GGATGAAGATGAAAGAGCTTATGCCACTATGTTGAGATGGTGCATGCATCATTACTCTGTGTATGCCGAGAAGGTTCACTTGCTGG				
GGATGAAGATGAAAGAGCTTATGCCACTATGTTGAGATGGTGCATGCATCATTACTCTGTGTATGCCGAGAAGGTTCACTTGCTGG				
D E D E R A Y A T M F E M V R C I I T L C Y A E K V H F A G				
1621	1641	1661	1681	1701
ATTCACTGCGCCGGCTCGCAGAGTGGAGAAGTTATTAACTTGCTGCCACGTATGTCAGATGTGGATGGAGTAT				
ATTCACTGCGCCGGCTCGCAGAGTGGAGAAGTTATTAACTTGCTGCCACGTATGTCAGATGTGGATGGAGTAT				
F T A P V C E S G E V I N L A A R M S Q M W M E Y K G N S A				
1721	1741	1761	1781	
AGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGCGGTTCGAAGGTAAGCCTATCCCTAACCCCTCCCTCGGCTCGATTC				
D I Q H S G G R S S L E G P R F E G K P I P N P L L G L D S				
1801	1821			
TACCGTACCGGTATCATCACCATCACCATGTA				
T R T G H H H H H H H -				

Upper nucleotide sequence = PCR consensus sequence
 Lower nucleotide sequence = clone 27 sequence
 Upper amino acid sequence = PCR consensus sequence
 Lower amino acid sequence = clone 27 sequence

Start codon (ATG) = nt 41-43
 nt 1-6 GCTAGC = NheI restriction site
 Nucleotide changes from the consensus sequence and the amino acids encoded
 - GAA and GAG both code for glutamic acid (E)

Cysteine residues (aa. 337 & 340) important for tubule formation (Monastyrskaya et. al., 1994)
 End of insert/gene = 1696
 Fusion peptide (V5 & 6xHIS) = nt 1696-1831
 Stop codon for fusion protein expression = nt 1832-1834 (TGA)

Figure 4. 8: The nucleotide sequence and the amino acid sequence for which it codes of BTV Seg-5, isolate RSAvvv/16.

The 5'UTR, the Seg-5 open reading frame and the fusion tag are shown. The cysteine residues that are critical for tubule formation are highlighted. Numbers refer to the nucleotide (nt) sequence.

Different strains of *E. coli* were tested as some may be more prone to toxic inserts than others. DH5 α and ABLE C (Stratagene) strains were poor hosts for the RSAvvv/16 NS1 gene construct, although the plasmid was successfully propagated in BL21 DE3 cells. A maxiprep purification of pcDNA3.1 V5/HIS_NS1 was therefore prepared from these cells for use in further experiments.

An identical strategy to that used to generate pNS3-EGFP was also used in an attempt to produce a NS1-EGFP plasmid for the expression of NS1 fused to EGFP for live cell imaging. However, in line with the difficulties experienced cloning pcDNA3.1 V5/HIS_NS1, not a single clone was obtained containing the NS1/EGFP fusion gene, regardless of the different approaches, host cells and different insert : vector ratios used.

4.5.2: Expression of BTV NS1 during infection

To determine the distribution of 'wild-type' NS1 in infected mammalian cells, BHK-21 cells were grown on glass coverslips and infected with RSAvvvv/16. The infected coverslips were fixed at sixteen hours post infection with 4 % paraformaldehyde. The coverslips were immunolabelled with a primary α NS1 antibody, (Orab 69, Materials and Methods, section 2.25, Table 2.12) and examined by confocal microscopy.

NS1 was abundant At 16 hours post infection and could be seen dispersed throughout the cell, as punctate dots, or collected into larger masses. There was also a tendency for the NS1 protein to cluster towards the cell-nucleus, in a pattern reminiscent of the nuclear membrane (Figure 4.9). However, NS1 also appeared to accumulate specifically at points either side of the nucleus (Figure 4.9). This concurs with previous electron microscopy data where NS1 tubules were found to be clustered around the microtubule organising centre (MTOC, P. Monaghan, unpublished observations).

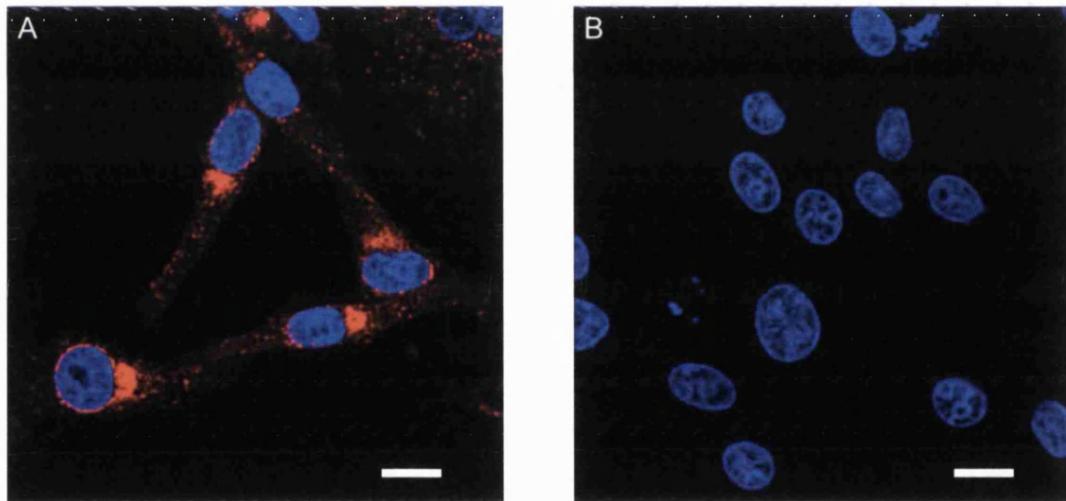


Figure 4. 9: NS1 distribution in infected BHK-21 cells.

BHK-21 cells were seeded on glass coverslips and infected (or mock infected) with RSAvvvv/16. At 16 hours post infection the cells were fixed using 4 % paraformaldehyde and immunolabelled using an anti-NS1 antibody. Nuclei were stained using DAPI (blue). The NS1 (red) was found to locate towards the nucleus and around the nuclear membrane in infected cells (A). Of then there was a particular accumulation to one side of the nucleus. NS1 labelling was not observed in uninfected cells (B)

Scale bar = 10 μ m

4.5.3: Transient transfection of pcDNA3.1 5/HIS_NS1 and confocal analysis

BHK-21 cells were seeded onto glass coverslips and grown for 24 hours, infected with RSAvvvv/16, then immediately transfected with pcDNA3.1 V5/HIS NS1, to allow expression of NS1-V5. At 24 hours post transfection the cells were fixed using 4 % paraformaldehyde and immunolabelled with the anti-V5 monoclonal antibody and an anti-NS1 polyclonal serum (α V5 mAb, Orab69, Materials and Methods section 2.25, Table 2.12) against the V5 epitope. Two distribution patterns were observed (Figure 4.10), in a manner similar to those seen for native NS1 during BTV infection, as described above (section 4.5.2 Figure 4.9).

These data indicate that the expressed V5 tagged NS1 behaves in a manner similar to native NS1 in terms of its distribution. This does not however demonstrate that its precise function is unaffected.

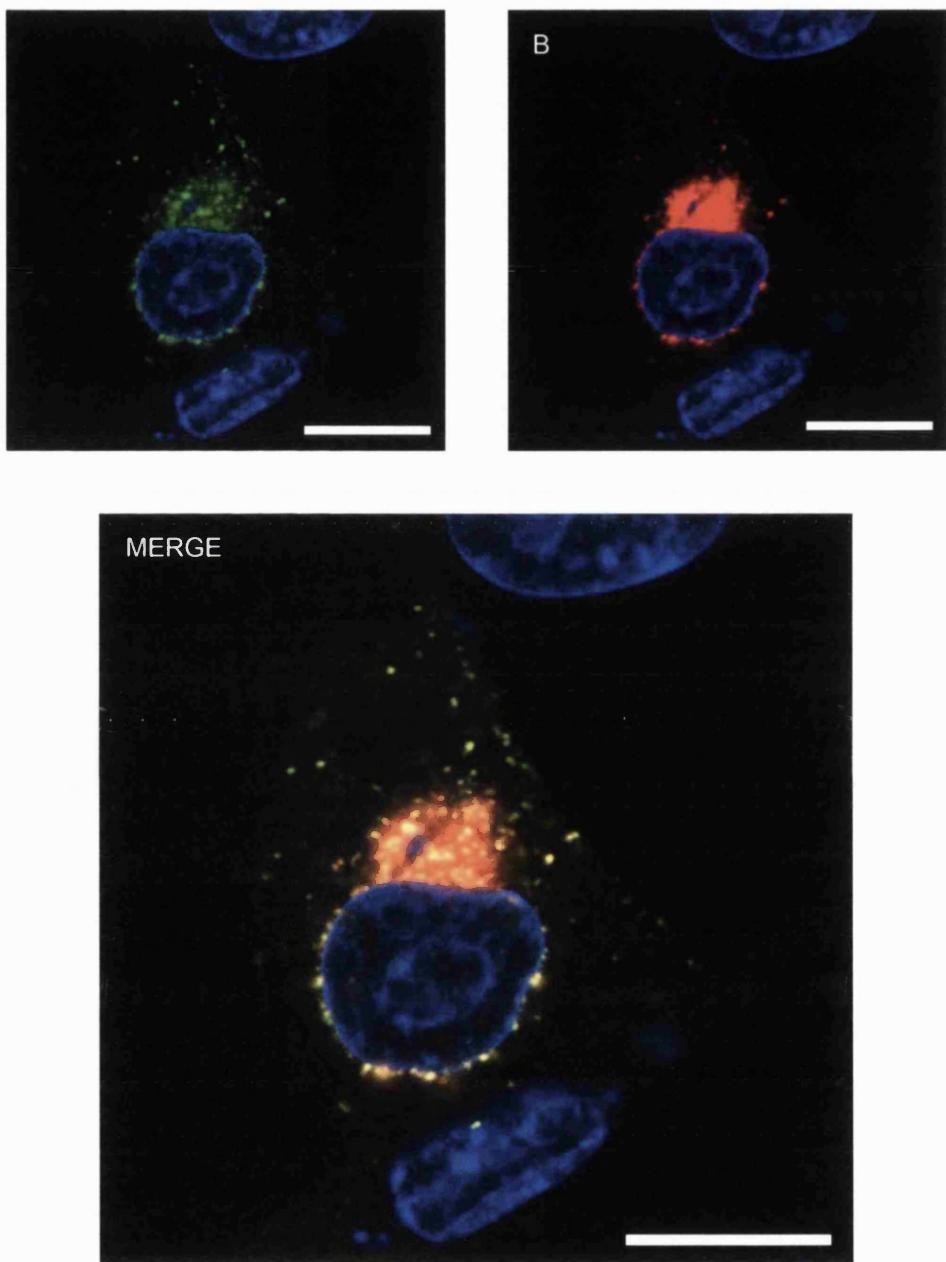


Figure 4. 10: The expression of NS1-V5 in mammalian cells.

BHK-21 cells were seeded on glass coverslips and 24 hours later infected with RSAvvvv/16. Immediately after infection the cells were transfected with pcDNA3.1V5/HIS NS1 and incubated for 24 hours. The cells were fixed using 4 % paraformaldehyde at 24 hours post-transfection and immunolabelled using antibodies to the V5 epitope and Orab 69 (Table 2.12, Chapter 2, Materials and Methods). Nuclei were stained using DAPI (blue). Expressed NS1-V5 (A, green) was found to co-localise with the native NS1 (B, red). The cytoplasmic dots, the nuclear membrane and a large perinuclear mass represented the major distributions of NS1.

Scale bar = 10 μ m

4.6: Transfection and Infection

4.6.1: Transfection and CPE

One of the underlying hypotheses that lead to the initiation of this project, is that the ratio of expression of BTV NS1 to NS3/3A determines the outcome of infection (Owens et al., 2004). It was suggested that increasing the level of NS3/3A expression relative to that of NS1, would switch the outcome of infection from lytic to persistent in mammalian cells.

In order to test this hypothesis, BHK cells growing in 24 well plates were infected with RSAvvvv/16 for 30 minutes at room temperature then transfected with plasmids that would express NS1-V5 or NS3/3A-V5. The cells were then incubated at 37 °C and monitored for cytopathic effect (CPE). Transfection of these plasmids into the cells had no visible impact on the outcome of infection. All of the infected wells showed equivalent levels of CPE throughout the course of infection, with 100 % CPE achieved after approximately 24 hours post infection (results not shown). CPE was largely absent in all of the uninfected wells. The only uninfected cells that gave any signs of marginal cytopathic effects were in control wells transfected with pcDNA3.1 V5/HIS_NS3/3A. These results were repeated with similar results, suggesting that rather than preventing CPE, NS3/NS3A may itself cause some cytopathic effects in mammalian cells. However, it is important to note that this protein is normally only synthesised in very small amounts, if at all in mammalian cells (e.g. BHK-21 cells) (Mertens et al., 1984).

4.6.2: Synthesis of BTV proteins from transfected plasmids, in cells BTV infected

Radiolabelling data (Figure 4.1) showed that shut-off of host cell protein synthesis is largely complete in BHK-21 cells, by 16 hours post infection with BTV.

The expression of proteins from transfected plasmids was examined in cells at the pre- and post shut-off phases of BTV infection. BHK-21 cells were transfected with pcDNA3.1V5/HIS_NS1, or pcDNA3.1 V5/HIS_NS3/3A for two periods during BTV infection: 0 – 12 hours and 12 – 24 hours post infection. At the end of each period the supernatant was discarded and the cells were harvested using 200 µl lysis buffer C. A 40 µl sample of the lysate was added to sample buffer and 15 µl analysed by SDS-PAGE. The gel was then western blotted using an α V5 mAb.

Expression of the NS1-V5 and NS3/3A-V5 was ‘robust’ during both periods in the uninfected but transfected ‘control’ cells, as detected by the western blot (Figure 4.11).

NS1-V5 was observed primarily as a single discrete band of the correct size (64 kDa), but with an additional lighter band of a slightly smaller mass. NS3/3A-V5 was observed as a large mass, comprising several bands on the gel.

The levels of NS1-V5 and NS3/3A-V5 expression from transfected plasmids were dramatically lower, in cells infected with BTV (RSAvvvv/16), during the early transfection period (0 – 12 hours, Figure 4.11). However, no fusion protein expression was detected in infected cells during the later transfection period (12-24 hours). These results demonstrate not only that BTV infection influences host cell processes involved in protein expression, but also that it has a suppressive effect on protein expression from these exogenous plasmids, despite the proteins themselves being derived from the same virus. These results were consistent across several experiments.

Although transcription from the transfected plasmids will generate mRNAs containing the BTV ORFs for NS3 or NS1, they have been engineered to express these as V5 fusion proteins, and have a 3' polyadenylate tail in place of the 3' non-coding regions present in the original BTV genome segments or mRNAs. They are therefore different from native BTV mRNAs, and these differences may explain their failure to be translated efficiently in BTV infected cells.

Subsequent studies (see Chapter 5) have indicated that both 3' and 5' untranslated regions (UTRs) are important for efficient translation of BTV mRNAs and are likely to help regulate BTV protein expression in infected mammalian and/or insect cells. The mRNAs transcribed from the plasmids may therefore be inefficient messages and their translation may also be shut-off, along with other non-viral (host-cell) mRNAs. These data also suggest that signals which ensure efficient translation of BTV mRNAs in infected cells (either early or late) are not primarily restricted in the coding region of these individual BTV genes.

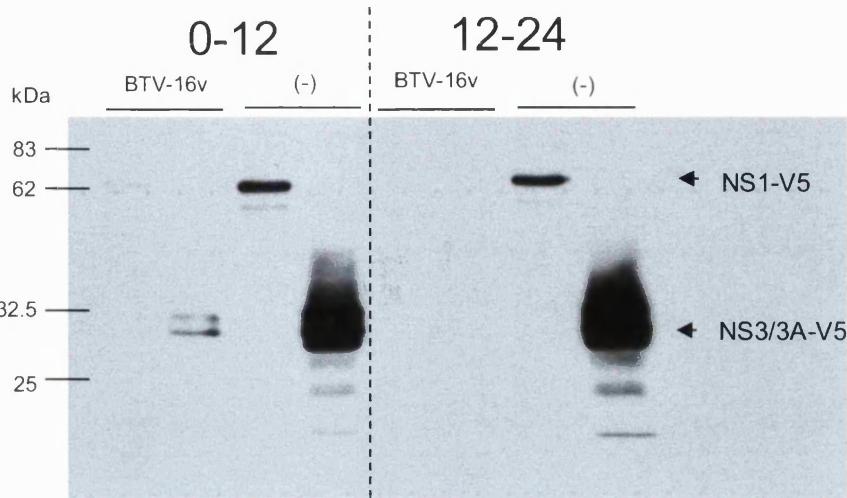


Figure 4. 11: Western blot analysis of fusion proteins expressed from plasmids in uninfected or BTV-16 infected BHK cells.

BHK-21 cells were either mock infected (-), or infected with RSAvvvv/16 (BTV-16v). Transfection complexes containing pcDNA3.1V5/HIS NS1 (expressing V5-NS1) or pcDNA3.1V5/HIS NS3/3A (expressing V5-NS3/3A) were prepared as detailed in Materials and Methods (section 2.3). These transfection complexes were added to the cells either immediately (0-12) or at 12 hours post-infection (12-24). The transfected cells were incubated for 12 hours and harvested in lysis buffer. The cell lysates were analysed by SDS-PAGE followed by western blotting using antibodies (α V5) that are specific for the V5 epitope tag. Good expression was observed for both transfection periods in uninfected cells. However, infection with BTV greatly reduced the expression of both NS1-V5 and NS3/3A-V5 from these plasmids, with the lowest expression levels in the second period (12-24 hours).

An attempt was made to determine the distribution of the proteins derived from transfected plasmids in infected cells, as compared to uninfected cells. BHK-21 cells were seeded onto glass coverslips 24 hours prior to infection. Cells were then infected with BTV, immediately transfected with pNS3/3A-EGFP, and fixed with 4 % paraformaldehyde 24 hours post infection/transfection. The coverslips were immunolabelled using an α NS2 antibody (Orab1 – Materials and Methods, Table 2.12) and analysed by confocal microscopy. In excess of 90 % of the cells were infected, as indicated by NS2 immunolabelling of VIBs. The NS3/3A-EGFP distribution was primarily Golgi-like and along the plasma membrane (Bansal et al., 1998; Hyatt et al., 1991) (Figure 4.12). However, with few exceptions, the cells expressing observable levels of NS3/3A-EGFP were either uninfected, or only very lightly infected (Figure 4.13). This may be explained by the shut off of plasmid derived translation observed in the previous section.

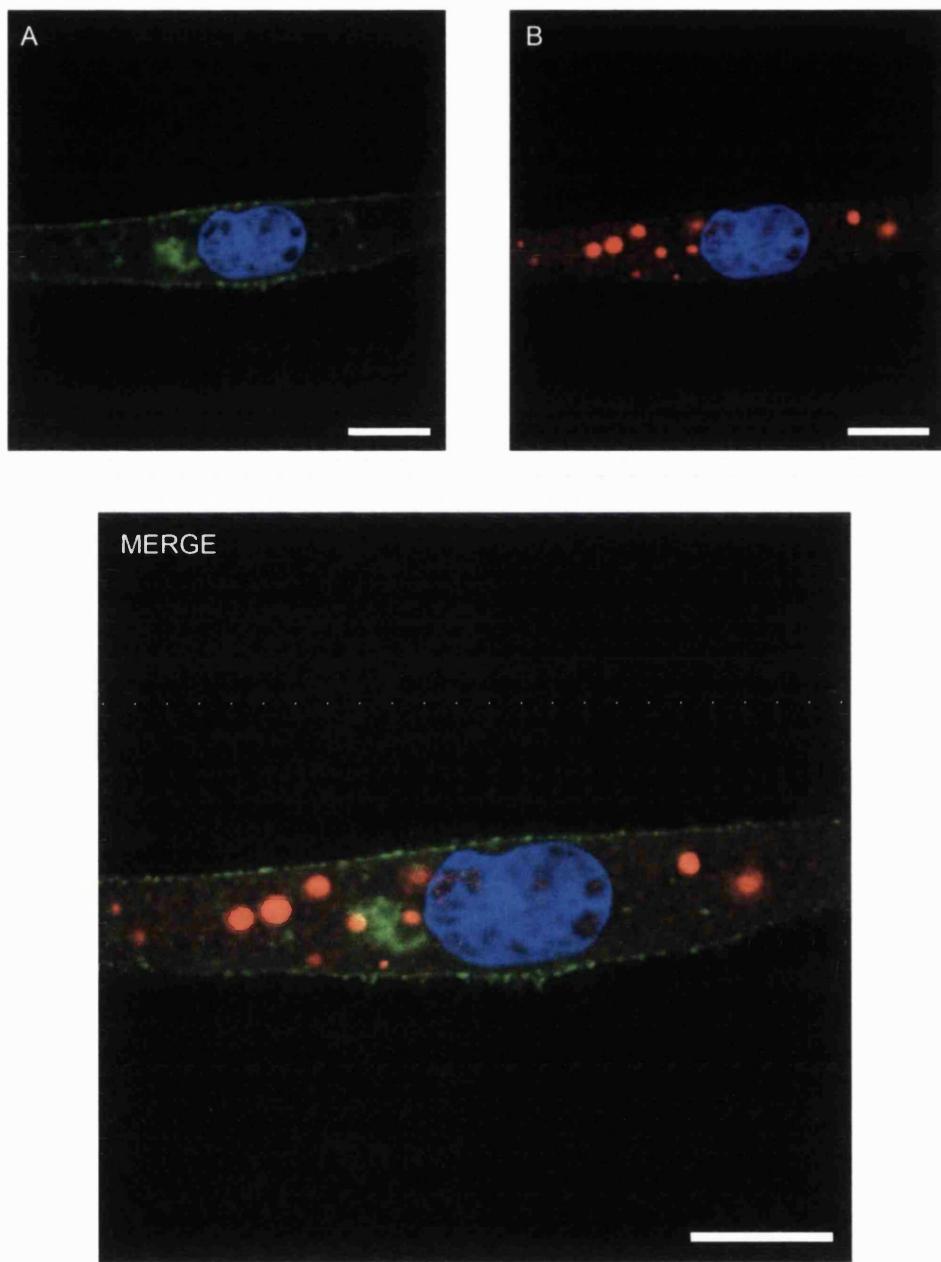


Figure 4.12: Expression of NS3/3A-EGFP in a BTV infected cell.

BHK-21 cells were seeded on glass coverslips and 24 hours later infected with RSAvvvv/16. Immediately after infection, the cells were transfected with pNS3/3A-EGFP and incubated for 24 hours. The cells were fixed using 4 % paraformaldehyde and immunolabelled using an anti-NS2 antibody (Orab 1, Table 2.12). Nuclei were stained using DAPI (blue). NS3/3A-EGFP (green) demonstrated the expected distribution in the Golgi and plasma membrane (A) whereas NS2 (red) formed characteristic viral inclusion bodies (B). The merged image indicates that the proteins did not appear to co-localise (C). The NS3/3A-EGFP was more localised as distinct dots along the plasma membrane compared to uninfected cells (Figure 4.6).

Scale bar = 10 μ m

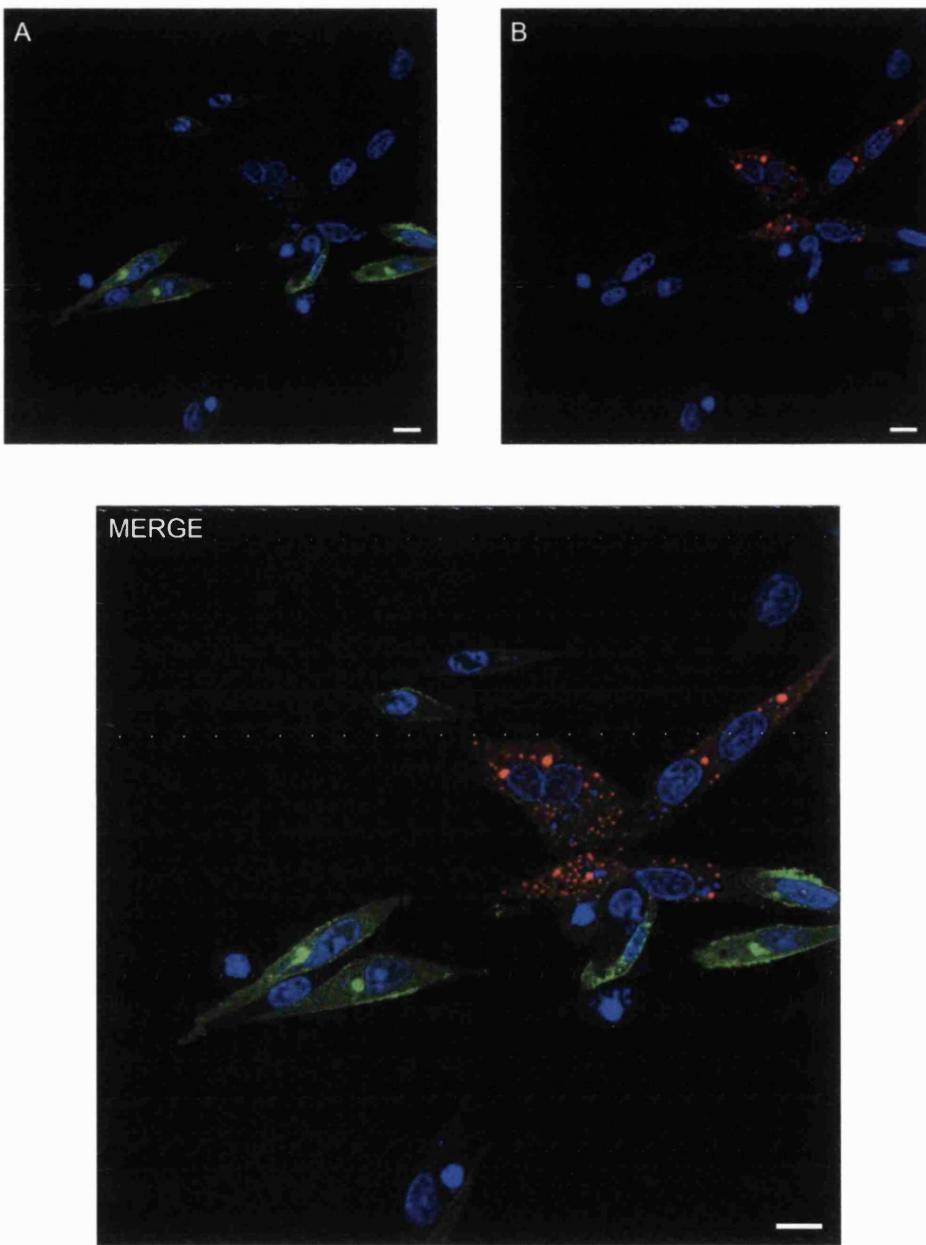


Figure 4. 13: Lack of apparent co-infection/transfection.

BHK-21 cells were seeded on glass coverslips and 24 hours later infected with RSAvvvv/16, and transfected with pNS3/3A-EGFP. The cells were fixed using 4 % paraformaldehyde at 24 hours post infection/transfection and immunolabelled using an anti-NS2 antibody (Orab 1, Table 2.12). Nuclei were stained using DAPI (blue). Expressed NS3/3A-EGFP (green) exhibited the expected distribution in the Golgi apparatus and along the plasma membrane (A). Infected cells contained NS2 in the form of viral inclusion bodies (red, B). However, expression from the plasmid was only occasionally observed in infected cells.

Scale bar = 10 μ m

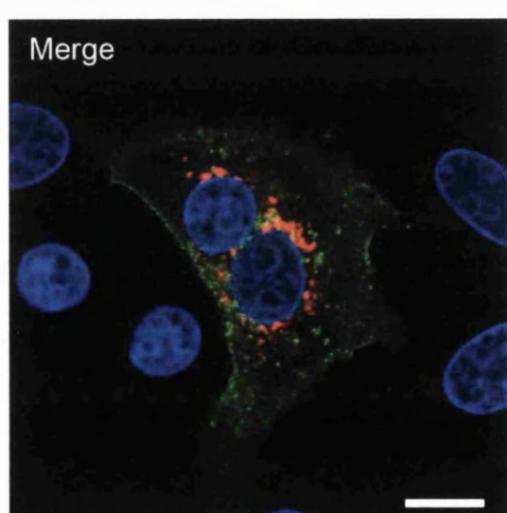
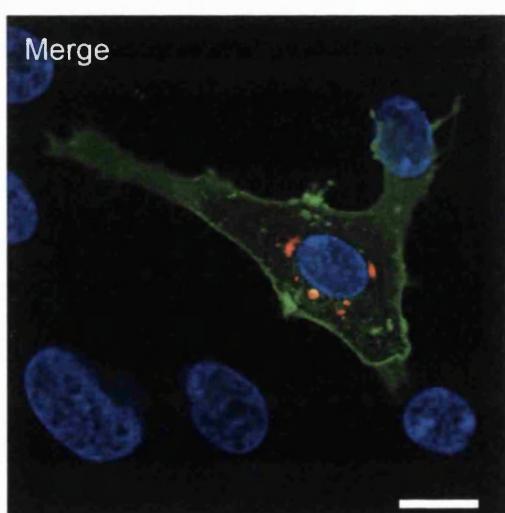
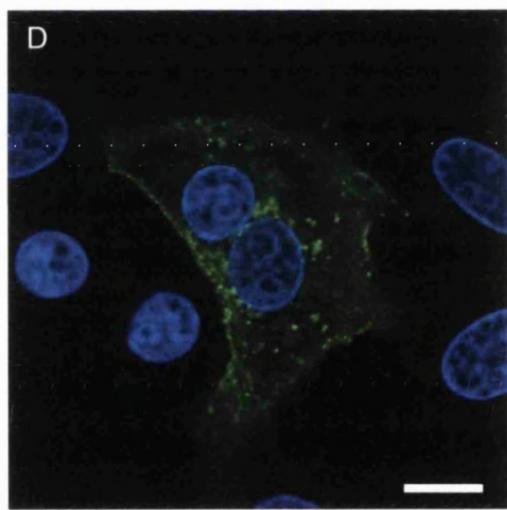
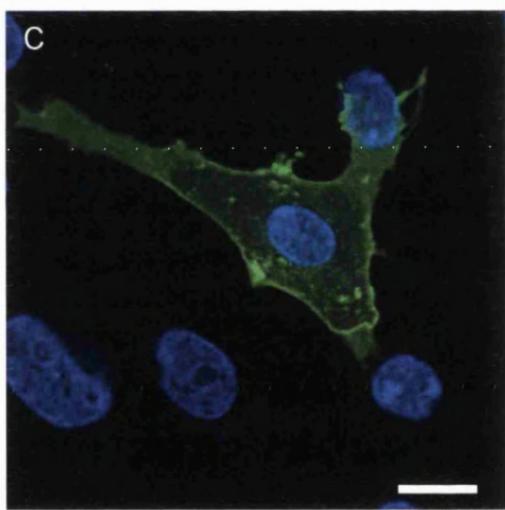
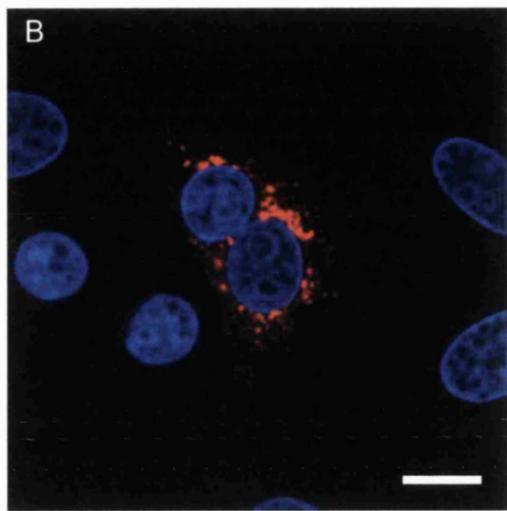
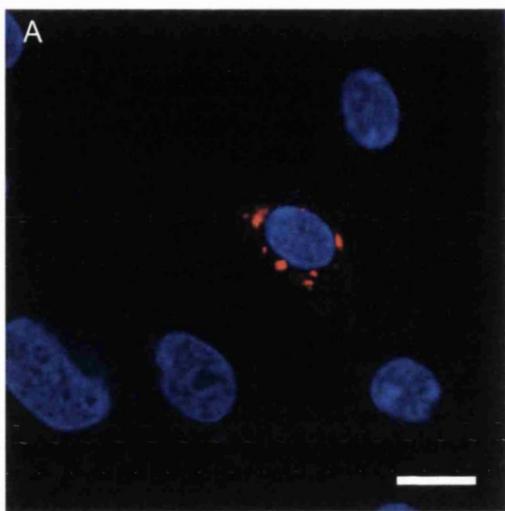
In a similar experiment where infected or uninfected cells were co-expressing both NS1-V5 and NS3/3A-EGFP, the distribution of either protein was not markedly altered by infection, (Figure 4.14), although the NS3/3A-EGFP in the infected cells was generally less evenly distributed along the membrane (Figure 4.14). In both cases the NS3/3A-EGFP localised to the Golgi and plasma membrane, and NS1 tended towards a perinuclear location. However, the lack of labelling for a virus derived protein meant that it was impossible to confirm that individual cells were infected.

[Figure on next page]

Figure 4.14: Co-expression of NS1-V5 and NS3/3A-EGFP in uninfected and infected cells.

BHK-21 cells were seeded on glass coverslips and 24 hours later infected (or mock infected) with RSAvvvv/16. After infection, the cells were co-transfected with plasmids pcDNA3.1V5/HIS_NS1 and pNS3/3A-EGFP. The cells were incubated for 24 hours to allow expression and then fixed using 4 % paraformaldehyde. The cells were immunolabelled using an anti-V5 antibody. Nuclei were stained using DAPI (blue). The images are represented of the pattern observed in all co-transfected cells. In both the uninfected (A) and infected (B) cells, NS1-V5 (red) was located as a perinuclear distribution. The intracellular distribution of NS3/3A-EGFP (green) appeared similar in uninfected (C) and infected (D) cells. However, in infected cells the NS3/3A-EGFP appeared as small aggregates along the plasma membrane in contrast to an even distribution in uninfected cells.

Scale bar = 10 μ m



4.7: RNA silencing

Two approaches were used to generate dsRNA substrates for digestion by Dicer:

- Synthesising dsRNA using phi6 polymerase using a ssRNA template
- *In vitro* transcription of the sense and anti-sense ssRNA strands followed by annealing the strands together.

In both cases the ssRNA transcripts were initially generated by T7 *in vitro* transcription from cDNA PCR amplicons containing the T7 promoter.

dsRNA production part 1: Phi6 polymerase

4.7.1: RT-PCR amplification (the T7 promoter and the phi6 sequence)

RT-PCR products for *in vitro* transcription were produced using primers containing the core T7 promoter sequence [TAATACGACTCACTATA] positioned 5' relative to sequences targeting the terminal sequences of segments 5 or 10 (Table 4.1). The T7 sequence was only added to one end of a RT-PCR product; the other end of the product matched the terminal sequence of the segment. The efficiency of T7 polymerase is increased by the presence of guanosine residues as the initial bases in the transcript (Patnaik et al., 1992). Two additional guanosine bases were therefore inserted between the core T7 promoter and the end of the BTV segment, resulting in three guanosines as the first transcribed bases (Figure 4.15).

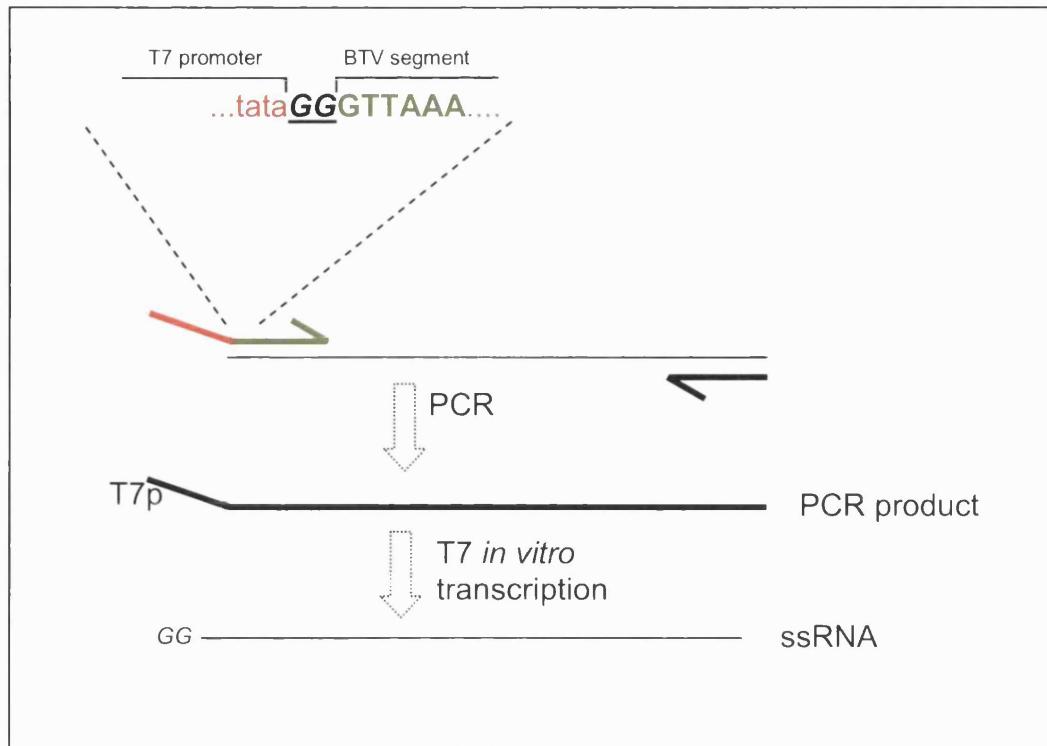


Figure 4. 15: Illustration of procedures used for *in vitro* synthesis of ssRNA from BTV segments.

The core T7 promoter (T7p) was positioned at the 5' end of a primer targeting the desired sequence that was amplified by PCR from viral RNA that had been reverse transcribed using MMLV. The T7p was therefore incorporated into the resulting PCR product used as the DNA template for *in vitro* transcription reactions. Three guanosine bases are incorporated immediately after the T7p to ensure the efficiency of the T7 polymerase transcription. The addition of guanosine residues results in the presence of two non-BTV nucleotides at the 5' of ssRNA transcripts.

The efficiency of the phi6 polymerase is reportedly greater when the sequence [NNUUUUUUUUUCC] is included at the end of the product (Makeyev and Bamford, 2000a). This is the native phi6 polymerase ‘attachment’ sequence. PCR products with and without this sequence were generated for use in phi6 polymerase reactions by incorporating the sequence into the PCR primers.

Name	Sequence
T7ggSeg5(sense)	ACACT <u>TAATACGACTCACTATA</u> GGGTAAAAAGTTCTCTAGTTG
T7gg+optimal Seg5(sense)	ACACT <u>TAATACGACTCACTATA</u> GGGTTTTTTCC <u>GTTAAAAAGT</u> <u>TCTCTAGTTG</u>
T7ggSeg5(antisense)	ACACT <u>TAATACGACTCACTATA</u> GGG <u>TAAGTGAAGTTCTAGTA</u> <u>G</u>
T7ggSeg10(sense)	CAC <u>TAATACGACTCACTATA</u> GGG <u>TTAAAAAGTGTGCGCTGCC</u>
T7gg+optimal Seg10(sense)	ACACT <u>TAATACGACTCACTATA</u> GGGTTTTTTCC <u>GTTAAAAAGT</u> <u>TCGCTGCC</u>
T7ggSeg10(antisense)	ACACT <u>TAATACGACTCACTAT</u> AGGG <u>TAAGTGTGTAGTATCGCG</u>
S10 1-20F	GTTAAAAAGTGTGCGCTGCCA
BTV-16v Seg5 3'+optimal R	GGAAAAAAAAGTAAGTTGAAAGTTCTAGTAGAGTGC
BTV-16v Seg10 R	GTAAGTGTGTAGTATCGCG
BTV-16v Seg10 3'+optimal R	GGAAAAAAAAGTAAGTGTGTAGTATCGCG
BTV-16v Seg5 R	GTAAGTTGAAAGTTCTAGTAGAGTG
S5 1-20F	GTTAAAAAGTTCTCTAGTTGGC

Table 4. 1: Primer sequences production *in vitro* transcription templates by PCR.
 These primers were used to amplify Seg-5 or 10 sequences and incorporate a T7 promoter so that the resulting amplicons could be used as templates for *in vitro* transcription reactions. The core **T7 promoter sequence**, **phi6 optimal sequence** and **viral sequences** are highlighted.

The high fidelity DNA polymerase ‘KOD’ was used for all PCR reactions in order to minimise the frequency of errors incorporated into the template for *in vitro* transcription. PCR was performed using appropriate primers and RSAvvvv/16 cDNA reverse transcribed using MMLV as a template (Table 4.1 and Materials and methods sections 2.7 and 2.8.2). The reaction was analysed by AGE and the PCR product gel extracted. This PCR product was then used as a template for *in vitro* transcription.

4.7.2: *In vitro* transcription of ssRNA

Single stranded RNA (ssRNA) representing the sense strand of the BTV genome was generated using the Megascript T7 *in vitro* transcription kit (Ambion). Transcription reactions contained 200 ng of RT-PCR product. After two hours incubation at 37°C, the DNA template was digested by incubating the reaction with 1U of TURBO DNase for 15

minutes at 37°C. The ssRNA was purified using the RNA clean-up protocol of the RNesay Mini kit (Qiagen) and used as the template for phi6 polymerase reactions.

4.7.3: Phi 6 Reactions

Phi6 polymerase can synthesise ssRNA from a dsRNA template, and dsRNA from an ssRNA template (Makeyev and Bamford, 2000b). As an initial pilot, reactions were assembled as described in the Materials and Methods (section 2.26) and incubated for 2 hours using 1 unit of Phi6 polymerase before the reaction was analysed by AGE on a 1.2% non-denaturing agarose gel. As a comparison, the equivalent mass of the starting ssRNA (without phi6 treatment) was run in adjacent lanes. In cases where the ssRNA had been incubated with phi6 polymerase, a band was visible at the appropriate size for the segment, implying that the complementary ssRNA was being synthesised, making double stranded RNA, which may have been further amplified (making more ssRNA) by the phi6 polymerase (Figure 4.16). This indicated that, with suitable scaling, the phi6 enzyme could be used to generate dsRNA as a precursor for cleavage into siRNAs.

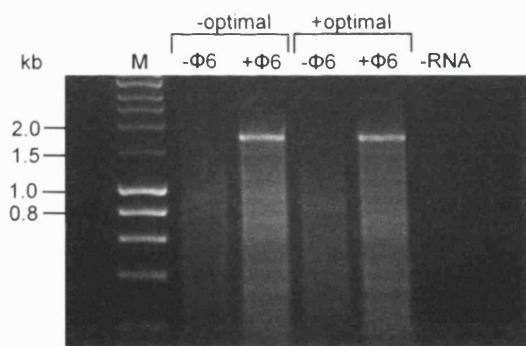


Figure 4. 16: Synthesis of dsRNA from ssRNA, using the Phi6 polymerase.
BTV-16 Seg-5 ssRNA (In vitro transcribed from cDNA amplicons) without (- optimal) or with (+ optimal) the phi6 terminal sequence was added to phi6 polymerase reactions and incubated at 32 °C for 2 hours. Samples of ssRNA which had not been incubated with the polymerase (-Φ6) were run alongside samples of the phi6 polymerase reactions (+Φ6). A band of the correct size was observed after incubating the reaction with phi6 polymerase. The addition of the phi6 sequence was not found to be beneficial to the efficiency of dsRNA synthesis.

4.7.4: Phi6 optimisation

A substantial amount of dsRNA would be required to generate sufficient siRNAs for the planned NS1 and NS3/3A silencing experiments. Attempts were therefore made to optimise the phi6 polymerase reactions.

4.7.5: Units of phi6 polymerase

To determine whether dsRNA production could be increased by adding more units of phi6 polymerase, reactions were assembled as above but with either one, two or three units of enzyme. The reactions were incubated for two hours and the products analysed by AGE. The additional units of enzyme had no effect on the amount of dsRNA produced Figure 4.17. This suggests that the rate limiting factor could be the amount of available template, or the amounts of available NTP substrate.

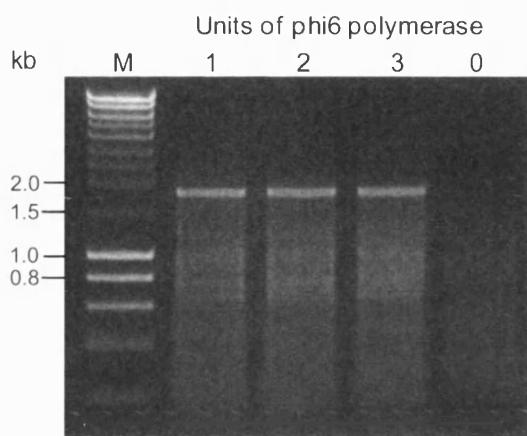


Figure 4. 17: The impact of phi6 polymerase concentration on dsRNA synthesis.
One microgram of Seg-5 ssRNA was incubated for 1 hour at 32 °C with 0, 1, 2 or 3 units of phi6 polymerase and appropriate substrates (Materials and Methods, section 2.26). The reaction products were analysed by AGE (Materials and Methods, section 2.10). The addition of extra units of phi6 polymerase failed to increase the intensity of the bands indicating that dsRNA synthesis had not been enhanced. The rate limiting factor may therefore be either the initial template

4.7.6: Incubation time

Incubating the reaction for additional time would conceivably allow increased production of the dsRNA. Bulk phi6 reactions were assembled and divided into two. Reactions were incubated for either two or four hours prior to analysis by AGE. The resulting band intensities suggest that a longer incubation time also has little or no effect on the final amount of dsRNA generated (Figure 4.18). The substrates should have been in excess, in the reaction, so the result also implies that the rate limiting factor is the original amount of template and that the phi6 polymerase is simply adding a complementary strand, converting ssRNA to dsRNA. A further observation from the gel is that the addition of the phi6 sequence to the RNA template, did slightly increase the reaction yield.

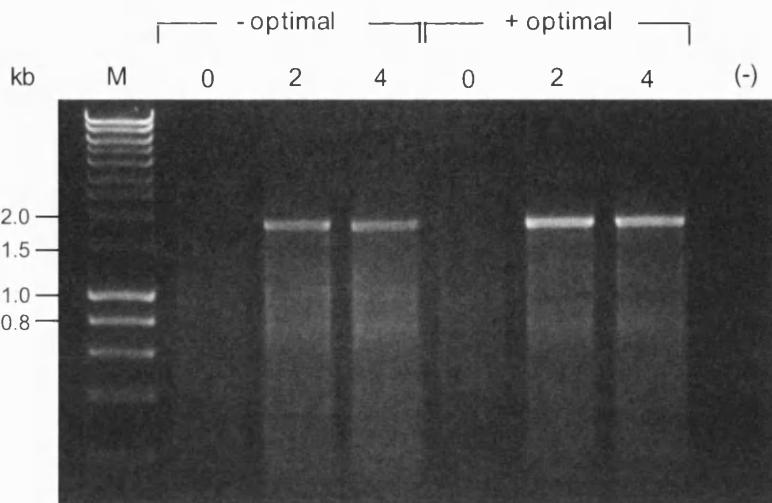


Figure 4. 18: Effect of Incubation time on dsRNA synthesis by phi6 polymerase.

One microgram of Seg-5 *in vitro* transcribed ssRNA without (-optimal) or with (+ optimal) the phi6 terminal sequence was added to phi6 polymerase reactions and incubated for 0, 2 or 4 hours at 32 °C and separated by AGE. No band was visible at 0 hours incubation whereas 2 and 4 hours incubation resulted in bands of the correct size for Seg-5. The increase in time made no difference to the band intensity and the addition of the optimal sequence slightly increased the band intensity.

The data obtained from these initial experiments suggest that more dsRNA could possibly be generated in much larger reactions (possibly without increasing the amount of enzyme used), or by using much larger amounts of ssRNA template, or both. However, the quantity of dsRNA that was produced using the phi6 polymerase was relatively small compared to the amounts that would be needed for Dicer digestion, to generate effective pools of siRNA. An alternative method was therefore also tested for dsRNA synthesis from specific BTV genes – *in vitro* transcription of both strands individually followed by annealing.

dsRNA production part 2: Large scale in vitro transcription and annealing

The T7 polymerase is a highly processive enzyme capable of synthesising large quantities of ssRNA in a short period of time. Using the T7 polymerase to synthesise large amounts of ssRNAs representing the two strands of a specific BTV genome segment, followed by annealing is a potentially rapid way to generate relatively large amounts of dsRNA.

4.8: RT-PCR

RT-PCR reactions were assembled to generate cDNA amplicons as templates for use as the substrates for the T7 polymerase *in vitro*. Two PCR products were therefore generated for each segment. One product, using primers T7ggSeg5(sense) and BTV-16v Seg5 R (Table 4.1), is identical to that made for the phi6 system (but without the phi6 sequence - section 4.7.1) allowing transcription of the sense strand from Seg-5.

Another PCR product was generated using primers T7ggSeg5(antisense) and BTV-16v TF (Table 4.1). This cDNA product allowed transcription of the antisense strand by T7 polymerase. Equivalent PCR products were also made for Segment – 10. All of the PCR products were gel purified and re-extracted.

4.9: Transcription, annealing and RNase A digest to eradicate ssRNA

The T7 RiboMAX Express RNAi System is a kit for the large scale *in vitro* transcription of dsRNA using the strategy employed here. The T7 transcription reactions were assembled containing 10 µl 2 × transcription buffer, 2 µl T7 enzyme and 1 µg of the appropriate PCR product (template) in a final volume of 20 µl. The reactions were incubated at 37 °C for 30 minutes. The DNA template was removed by adding 1 unit of DNase to the reaction and a further 30 minutes of incubation at 37 °C. For each genome segment, the reactions containing the sense and antisense strand were combined and heated to 70 °C for 10 minutes. The mixed reactions were then allowed to cool to room temperature for annealing to occur, before adding 2 µl of RNase A solution (20 µg/ml). The reaction was incubated at 37 °C for 30 minutes to allow the digestion of un-annealed ssRNA. The remaining dsRNA was precipitated using isopropanol and sodium acetate, washed once with 70 % (v/v) ethanol and resuspended in 100 µl of RNase free water. Half of the precipitated RNA was further purified using the RNA cleanup protocol of the RNeasy kit (Qiagen). One set of reactions using this Ribomax method yielded approximately 150 µg dsRNA per segment which was sufficient for the planned dicer treatments and silencing experiments.

4.10: dsRNA characterisation

Two experiments were performed to determine if the RNA resulting from the Ribomax procedure was double stranded. These experiments depend upon the properties of dsRNA, namely the more efficient migration of ds RNA in polyacrylamide gels, and

the resistance of dsRNA to digestion by RNase A in >0.3 M sodium chloride at (Dobos, 1976).

4.10.1: PAGE

It has previously been established that large ssRNAs migrate poorly in polyacrylamide gels, while each dsRNA species migrates as a tight band.

The silver stained gel showed that double stranded RNA had been produced using both methods of dsRNA synthesis. Dense bands were observed at the appropriate size for the segments, demonstrating that a large proportion of the synthesised RNA existed as full length segments (Figure 4.19). However, the RNA samples which had been synthesised using the phi6 polymerase (which had not been pretreated with ribonuclease A) also contained ssRNA, as revealed by a smear at the top of the lane, possibly indicating the synthesis of ssRNA by the phi6 polymerase. All of the RNA produced using the Ribomax kit entered the gel indicating that the RNase A treatment had removed all of the ssRNA in the sample. In all cases there was a smear of lower molecular weight bands. The Seg-10 sample produced using the Ribomax system also appeared to contain dsRNA fragments of a greater molecular weight than the size anticipated for Seg-10 (Figure 4.19). This result was somewhat unexpected, but may be explained by incorrect annealing of the strands and therefore an altered migration rate. However, the primary bands representing Seg-5 and Seg-10 both aligned well with the appropriate band of the RSAvvvv/16 RNA marker.

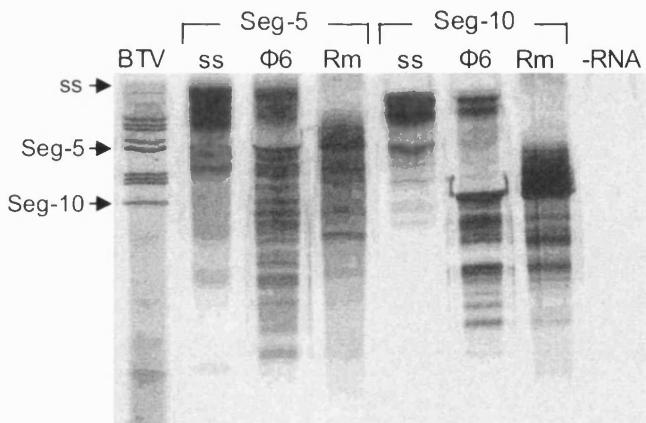


Figure 4.19: Confirmation of the ds nature of *in vitro* synthesised dsRNA by SDS-PAGE.

One microgram samples of single stranded (ss), the dsRNA generated using phi6 polymerase treatment ($\Phi 6$) or the dsRNA T7 Ribomax (Rm) were mixed with sample buffer (without DTT) in a final volume of 10 μ l. The samples were initially heated to 50 °C for 5 minutes, loaded into a 10 % SDS-PAGE gel, and separated at 200V for approximately one hour. Purified RSAvvvv/16 dsRNA (BTV) was used as a control marker for the segments. The gel was then silver-stained using the SilverSNAP stain kit (Pierce) and incubated at room temperature overnight in fix solution (30 % ethanol, 10 % acetic acid). The gel was developed using the kit reagents and then scanned. The results are similar for Seg-5 and Seg-10. The ss RNA entered the gel poorly, although a smear was generated. All of the samples produced using the Ribomax method entered the gel suggesting that these samples are double stranded. The samples subjected to phi6 polymerase treatment contained a dsRNA band of the correct size which entered the gel, but also contained ssRNA.

4.10.2: Ribonuclease A resistance in >0.3 M NaCl₂

Four RNase A digestion reactions were assembled, containing five micrograms of the synthesised dsRNA from either Seg-5 and seg-10, in a total volume of 100 μ l NaCl₂ solution, with final NaCl₂ concentrations of either 0.2 M or 0.5 M. A 10 μ l aliquot of each reaction was removed for gel analysis and 0.9 μ l of RNase A (1 μ g/ μ l) was added to the remaining 90 μ l. The reactions were incubated at 37 °C for 1 hour before analysis by AGE. The non-digested RNA from both NaCl₂ concentrations formed of a single strong band at the appropriate size for the segment (Figure 4.20).

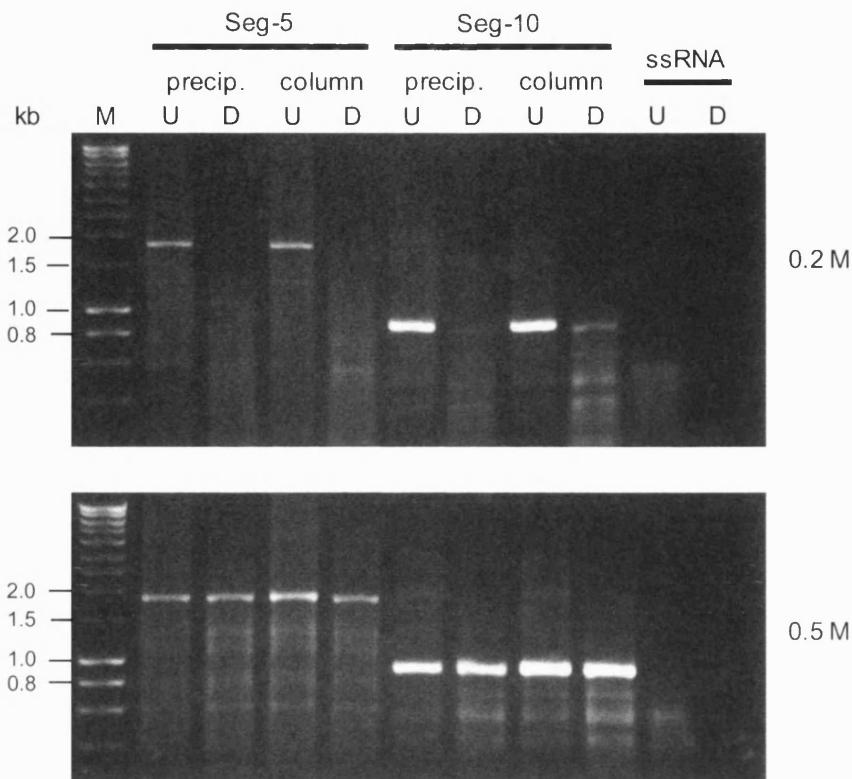


Figure 4.20: Confirmation of the ds nature of the *in vitro* synthesised dsRNA: RNase A digestion.

Five micrograms of the Seg-5 or Seg-10 dsRNA synthesised using the T7 Ribomax method was added to 100 μ l of 0.2 or 0.5 M NaCl₂. 10 μ l of the sample was removed as an undigested control (U). The remaining 90 μ l was incubated for 1 hour at 37 °C in the presence of 0.9 μ g of RNase A (D). In the presence of 0.2 M NaCl₂ the dsRNA samples and a ssRNA control was digested. The *in vitro* synthesised dsRNA was resistant to RNase A digestion in the presence of 0.5 M NaCl₂, confirming the double stranded nature of the RNA. The results were identical for dsRNA which had been purified using isopropanol precipitation (precip.) or isopropanol precipitation followed by the RNeasy clean-up protocol (column).

The RNA was digested by RNase A in 0.2M NaCl₂, forming a smear of RNA at a molecular weights lower than the intact segment, but the RNA was not digested in 0.5 M NaCl₂, (Figure 4.21). Single stranded RNA was also digested at both 0.2M and 0.5M NaCl₂. These results show that the Ribomax synthesised RNA is primarily double stranded. The additional purification using the RNeasy clean-up protocol had little if any effect upon the RNA profile, although this procedure removed unincorporated ribonucleotides and non-RNA contaminants.

Based on its productivity, ease of use and the ds nature of the RNA generated, the Ribomax method was chosen for large scale production of dsRNA using PCR products

amplified from reverse transcribed viral RNA and containing the T7 promoter. The BlockIT kit (Invitrogen) was used to cleave the resulting dsRNA and purify the resulting siRNAs (approximately 22 bp long) (Figure 4.21).

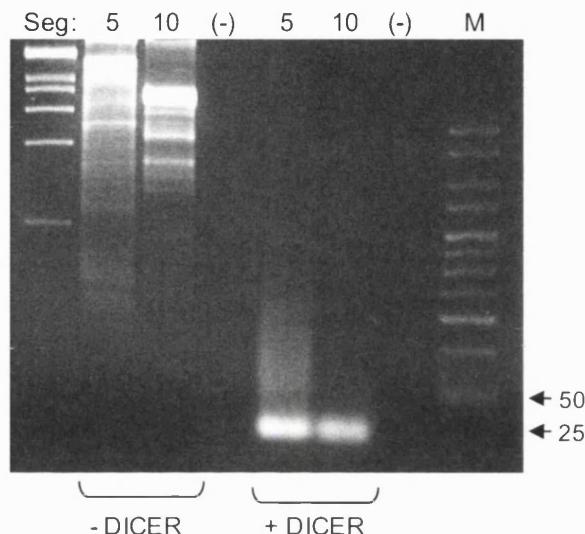


Figure 4. 21: Dicer digestion of *in vitro* synthesised dsRNA.

1 µg of the *in vitro* synthesised dsRNA segments representing Seg-5 (5) or Seg-10 (10) was digested and purified using the BLOCK-iT siRNA generation kit (Invitrogen). 100 ng of digested (+Dicer) or undigested (-Dicer) dsRNA was run on a 3 % agarose gel alongside negative controls. ‘M’ represents a 25 bp ladder. The full length segments (and the high molecular weight ladder) entered and migrated in the gel poorly (-DICER). In contrast, the siRNA pool migrated as a band of the correct size (20-25 bp, +DICER). Some smearing was evident for Seg-5, possibly indicating undigested RNA or aberrant migration of the siRNAs.

4.11: Optimisation of transfection conditions

siRNA delivery conditions were optimised in order to give the highest level of RNA silencing. TransIT-TKO and siQuest kits (both Mirrus) were tested for their ability to transfect BHK-21 cells with ‘glowing’ siRNAs (siGLO, Dharmacon) which fluoresce when stimulated by the appropriate wavelength. The efficiency of transfection was assessed using confocal microscopy. A siGLO concentration of 30 nM using 2.5 µl of TransIT-TKO reagent produced the best results and resulted in every cell appearing to be transfected (Figure 4.22).

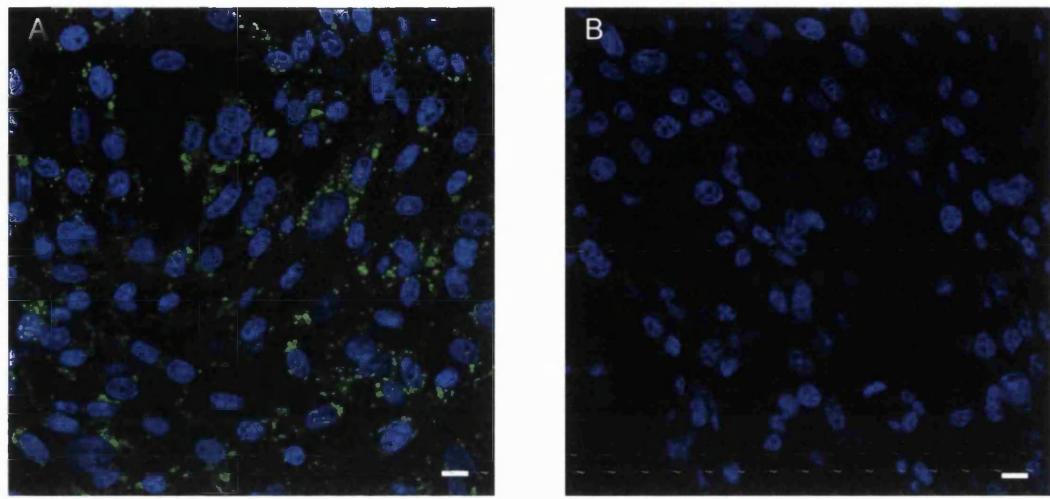


Figure 4. 22: Transfection of siGLO glowing siRNA.

BHK-21 cells were seeded on glass coverslips and incubated for 24 hours. The cells were transfected (or mock transfected) with siGLO at a final concentration in the media of 30nM. The cells were fixed using 4 % paraformaldehyde at 24 hours post transfection. The nuclei were stained using DAPI (blue). The siGLO (green) was evident in every transfected cell (A) whereas siGLO signal was absent in mock transfected cells (B). Scale bar = 10 μ m

4.12: Pilot silencing using a plasmid

The silencing of BTV NS protein expression, was assessed by co-transfected plasmids pcDNA3.1V5/HIS_NS1 and pcDNA3.1V5/HIS_NS3/3A, expressing epitope tagged versions of NS1 and NS3/3A respectively (sections 4.5.1 and 4.3.1), into uninfected BHK-21 cells, in the presence or absence of different amounts of the siRNAs. The cells in every well appeared healthy, with only slight signs of cytopathogenicity in the cells transfected with pcDNA3.1V5/HIS_NS3/3A. The cells were harvested in lysis buffer then analysed by SDS-PAGE and western blotting. NS1-V5 was expressed in the absence of siRNA but was significantly reduced when siRNA from Seg-5 dsRNA was also transfected into cells, although NS3/3A expression remained unaffected (Figure 4.23). The level of silencing did not appear to be highly dose-dependent, as the transfection of as little as 50 ng appeared to reduce expression. The initial experiments using siRNAs generated from Seg-5 dsRNA, suggest that this provides a potentially useful method for silencing target genes in BHK-21 cells. However, insufficient time remained in the project for this line of work to be completed.

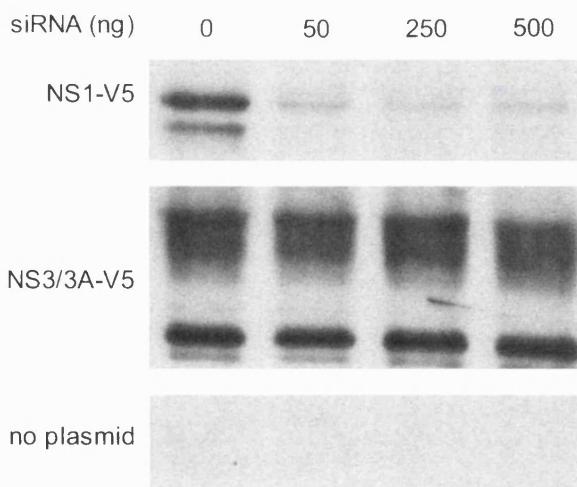


Figure 4. 23: RNA silencing of gene expression from a co-transfected plasmid.

BHK-21 cells were seeded 24 hours prior in 24 well plates so that 80 % confluence was achieved at the time of transfection. A series of co-transfection mixtures were prepared according to the instructions of TKO. Each transfection mixture comprised the NS1 or NS3/3A plasmids (pcDNA3.1V5/HIS_NS1 or pcDNA3.1V5/HIS_NS3/3A) with different amounts of the Seg-5 siRNA. For each well, 1.5 μ l of TransIT[®]-LT1 plasmid transfection reagent was diluted in 50 μ l of serum free medium (OptiMEM, Invitrogen) and incubated for 15 minutes at room temperature. Five hundred ng of the appropriate plasmid was added to the diluted transfection reagent and incubated for a further 15 minutes, before 2.5 μ l of TransIT-TKO siRNA transfection reagent was also added. After 15 minutes incubation, 0, 50, 250 or 500 ng of the Seg-5 derived siRNA was added to the

mixture the complete mixture was then incubated for 15 minutes. All but 250 μ l of the growth media was removed from the well, before the transfection mixture was added dropwise to the cells, which were then incubated at 37 °C for 24 hours. At 24 hours post co-transfection. the cells were harvested followed by SDS-PAGE and western blotting using an α V5 mAb to detect V5-tagged proteins. A significant reduction in pcDNA3.1V5/HIS NS1 expression was observed in the presence of the siRNA. In contrast no reduction was observed in the level of expression from pcDNA3.1V5/HIS NS3/3A.

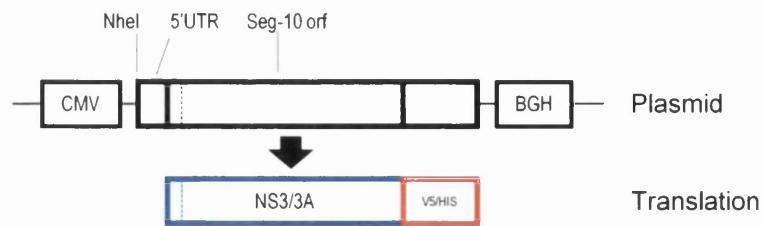
4.13: The University of London School of Pharmacy (ULSoP)

As part of a collaboration with The University of London School of Pharmacy (ULSoP) investigating novel methods of siRNA delivery for therapeutic purposes, the Ribomax method was used on a large scale to generate over 3 mg of dsRNA representing Seg-5 of the RSAvvvv/16 vaccine strain. This amount would not have been easily produced using the phi6 system. The dsRNA representing the full length of Seg-5 and plasmid pcDNA3.1 V5/HIS_NS1, were sent to ULSoP for further experiments outside of this project, relating to the delivery mechanism of siRNAs for therapeutic purposes.

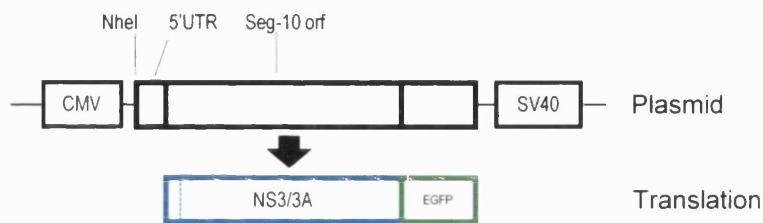
4.14: Discussion

This chapter reports attempts to alter the relative levels of BTV NS1 or NS3/3A expression in order to test the hypothesis that the ratio of NS1:NS3/3A determines the outcome of infection. The relevant genes were cloned into mammalian expression plasmids containing a CMV promoter to drive transcription within transfected cells (Figure 4.24). This allowed the relative amounts of NS1 or NS3 to be increased by intracellular expression. RNA silencing, using siRNAs, generated ‘in-house’ by ‘Dicer’ digestion of dsRNA, was used in an attempt to reduce the level of protein expression.

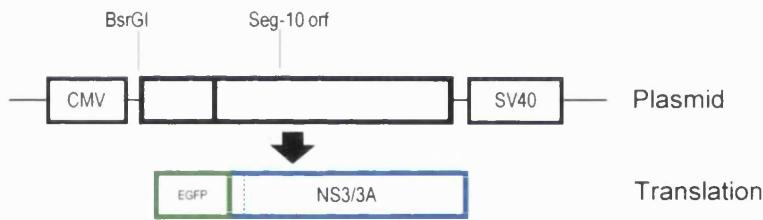
A)



B)



C)



D)

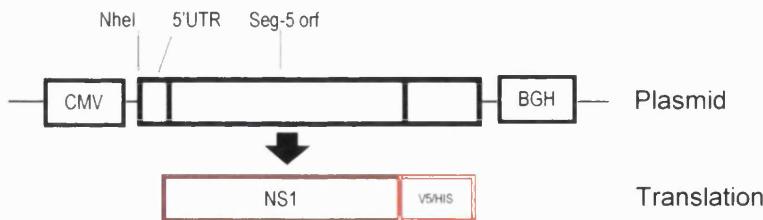


Figure 4. 24: The constructs assembled for the expression of BTV NS1 and NS3/3A. Four plasmid constructs were assembled for this part of the project. The 5'UTR and open reading frame of segment-10 was inserted into the cloning site of pcDNA3.1V5/HIS allowing the expression of NS3/3A with C-terminal V5 and 6HIS epitope tags (A). The NS3/3A sequence was also inserted at either the C terminus (B) or N-terminus (C) of EGFP in the plasmid pEGFP-N1 allowing the expression of NS3/3A with EGFP fused to either the C- or N-terminus of NS3/3A respectively. The 5'UTR and open reading frame of segment-5 was inserted into the cloning site of pcDNA3.1V5/HIS allowing the expression of NS3/3A with C-terminal V5 and 6HIS epitope tags (D). Drawings not to scale. BGH = bovine growth hormone poly adenylation signal. SV40 = SV40 poly adenylation signal. CMV = cytomegalovirus promoter.

The NS3/3A gene was readily clonable, allowing expression plasmids to be constructed with either a 45 amino acid sequence (containing V5 and His tags) fused to the carboxy terminus of NS3/3A, or with EGFP fused to either the amino or carboxy termini. The functional significance of the amino acid sequences present in NS3/3A were not investigated in detail during this project. The hydrophobic nature of the immediate N terminus is intriguing given the membrane associated nature of NS3/3A. No signal peptide has yet been reported for NS3/3A and thus it would be interesting to mutate these bases and observe the effects this may have on endoplasmic reticulum/Golgi localisation. Although a yeast two hybrid analysis has previously been performed using NS3/3A (Beaton et al, 2004), the presence of epitope tags at the C terminus would facilitate the pull down by immunoprecipitation of host proteins interacting with NS3/3A during further analysis of mutated versions of NS3/3A. Additionally, the highly charged region (amino acids 8-23) identified in the sequence was not investigated during this project. It would be interesting to alter the charged nature of this region and, in particular, mutate selected bases in a sequence of positively or negatively charged residues, e.g. glutamic acid at position 11.

The distribution of plasmid derived NS3/3A fusion proteins was analysed by confocal microscopy, indicating associations with the plasma membrane, Golgi and smooth-surfaced cytoplasmic vesicles. This distribution matches previously published observations (Bansal et al., 1998; Hyatt et al., 1991).

Live cell imaging indicated that the distribution of NS3/3A-EGFP and EGFP-NS3/3A around the cell was static rather than dynamic. Although subsequent immunolabelling for NS2 indicated virtually every cell was infected, subsequent results showed that the BTV proteins were poorly expressed from plasmids in infected cells and relatively few co-infected/transfected cells were detected. The cells in which the transfected proteins were detected by Live cell imaging, may therefore have been uninfected.

Confocal microscopy of co-infected/transfected cells indicated that the distribution of expressed NS3/3A was slightly altered in infected cells and appeared to be less uniformly distributed over the plasma membrane. This result agrees with observations by electron microscopy (P. Monaghan, unpublished), and data suggesting that NS3/3A interacts with other BTV proteins / factors, acting as a viroporin and resulting in the localised release virus particles (Han and Harty, 2004). The addition of EGFP to either end of the NS3/3A protein had no obvious effect on its distribution.

However, yeast two hybrid analyses by Beaton *et al* (2002) indicate that NS3/3A interacts with calpactin via its amino terminus and the presence of a protein greater in size than NS3/3A itself (EGFP) might be expected to hinder these interactions.

The expression of NS3/3A in uninfected cells appeared to cause mild cytopathic effect. This result appears to be contradictory to the hypothesis that high levels of intracellular NS3/3A results in a non-cytopathic infection, as observed in insect cells or as reported in cells where NS1 tubule formation has been disrupted (Owens et al., 2004). However, the NS3/3A protein of African Horse Sickness virus has been reported to be a pathogenicity factor in mammalian cells (Meiring et al., 2009; van Niekerk et al., 2001; van Staden et al., 1995).

In contrast the NS1 gene was refractory to cloning, and despite many attempts only a single clone was obtained. The intracellular distribution of expressed NS1-V5 as observed by confocal microscopy appeared to be similar to that of the native protein in infected cells. This suggests that F89S mutation has little if any impact on tubule folding or location. These data are in agreement with previous research which has demonstrated that tubule formation is dependent on the amino and carboxy termini as well as cysteine residues 337 and 340 (Monastyrskaya et al., 1994).

The transfection of plasmids to overexpress NS3/3A in infected mammalian cells failed to cause a transition from cytopathic to persistent infection, as was predicted in the hypothesis of Owens et al (2004). However, confocal microscopy indicated that the transfection efficiency was low and a high proportion of the cells were unaffected. In addition expression of BTV proteins from transfected plasmids was dramatically inhibited in BTV infected cells, particularly late in infection. If insufficient cells are transfected and producing large amounts of protein, then any effect may also be masked by the presence of untransfected cells. This makes it difficult to draw firm conclusions about the hypothesis by Owens et al. (2004).

The development of an inducible cell line, and/or the expression of BTV fusion proteins from plasmids/RNAs that have both BTV mRNA terminal regions at the appropriate positions (see Chapter 5), may produce higher levels of protein production in a higher percentage of infected cells. This approach may lead to more conclusive results. An investigation into the mechanism behind the inhibition of expression forms the basis for the next chapter.

The commercially available phi6 polymerase is a modified version of the phi6 bacteriophage P2 protein (Makeyev and Bamford, 2000b). This RNA dependent RNA

polymerase (RdRp) utilises either ss or ds RNA as a template (Makeyev and Bamford, 2000a; Makeyev and Bamford, 2000b). It is highly processive and provides an effective and simple method to generate large amounts of siRNA (Aalto et al., 2007), and was used in a first attempt to generate dsRNA prior to digestion using Dicer. However, the results obtained suggested that the activity of the enzyme was primarily to add a complementary strand to the input ssRNA. Despite attempts to optimise the reaction, SDS-PAGE analysis showed a substantial amount of ssRNA post phi6 treatment. The ssRNA may originate from unprocessed input ssRNA or from the phi6 polymerase activity in a manner similar to that previously reported (Makeyev and Bamford, 2000b). The ssRNA could be removed by treating the RNA with RNase A in the presence of >0.3 M NaCl₂ prior to purification.

A second method for the production of dsRNA, synthesised both strands independently (by T7) before annealing them together. The Ribomax kit for dsRNA synthesis was found to be efficient. The ssRNA could be specifically removed using RNase A in 0.5 M NaCl₂. The resulting RNA was successfully used for production of siRNAs with the capacity to silence protein expression from a plasmid. A good silencing response was observed when siRNA was co-transfected with a plasmid expressing the target protein. A slight ‘remaining band’ was detected, possibly representing protein synthesised in the period between transfection and the initiation of silencing. This could potentially be overcome by transfecting the siRNA 24-48 hours prior to the introduction of the target RNA.

‘Off target effects’ are an important consideration when using RNA silencing (Svoboda, 2007). The use of up to 1741 different siRNAs from the digestion of Seg-5 dsRNA (based on the 1763nt length of Seg-5 [BTV-16 ref strain] and 22 bp siRNAs [1763-22]) potentially increases the risk of experiencing off-target effects. Despite this concern, no silencing of the NS3/3A-V5 message was observed when Seg-5 derived siRNAs were co-transfected with pcDNA3.1 V5/HIS_NS3/3A.

It would perhaps be prudent to analyse the level of specificity achieved using this approach, as part of any further experiments. However insufficient time remained in the project to complete studies on reducing the level of NS1 via silencing of Seg-5 mRNA. The high level production of dsRNA could potentially result in improvements for delivery of siRNAs for therapeutic purposes using nanoparticles as described by (Katas and Alpar, 2006; Katas et al., 2009).

Chapter 5: The Translation of BTV and host mRNAs during BTV Infection

5.1: Introduction

After cell-attachment and entry, parental BTV-core-particles are released into the cytoplasm of the host-cells, where the core - associated TCs transcribe, cap and methylate mRNA copies of all ten genome segments. These mRNAs are extruded from the core through pores located at the 5-fold apices (Diprose et al., 2001) and provide a basis for BTV gene expression that is independent of cellular RNA-polymerase functions. The BTV mRNAs possess a (m⁷)GpppG(2O'm) 'type 1 Cap', similar to that of cellular mRNAs, allowing efficient binding of the cap-binding protein and eukaryotic translation initiation factor eIF4E (Ramadevi et al., 1998a; Sutton et al., 2007). Unlike BTV mRNAs, eukaryotic mRNAs also have a polyadenylated (poly(A)) tail, which binds to the poly-A binding protein (PABP), enhancing interactions with the translation initiation complex and the level of translation (Wells et al., 1998). In contrast, BTV mRNAs have 3'-untranslated-regions (UTR), which include six terminal nucleotides that are conserved in the mRNAs transcribed from each segment of the BTV genome (Mertens and Diprose, 2004b; Rao et al., 1983). Although the 3'UTRs are relatively highly conserved for the same segment but derived from different BTV strains, they do vary in both sequence and length (from 31 to 116 nucleotides), between the different BTV genome segments.

During BTV infection of most mammalian cells, host cell protein synthesis is 'shut-off', presumably releasing both substrates and ribosomes for translation of viral mRNAs and synthesis of viral proteins (Huismans, 1971). The mechanisms involved in host-cell shut-off could involve either a reduction in the availability of host mRNAs, and/or virus induced changes that result in a reduction in the relative efficiency of their translation. However, in order for the virus to take advantage of such changes, the efficiency of viral mRNA transcription and translation must remain largely unaffected, or even increased by such changes. Differences in the transcription mechanism and in the structure/sequence of the mRNAs that are generated by the host cell and the virus could therefore be important factors controlling the relative levels of viral and host gene expression.

It is reported in Chapter 4 that BTV infection has an inhibitory effect on the expression of proteins from transfected plasmids. It was concluded that the same factors affecting host protein shut-off, were also acting on 'mammalian-like' expression from

these plasmids. The cloned cDNA inserts in these plasmids contain the viral 5' UTR from segments 5 and 10). The most obvious difference between either host-cell mRNAs, or mRNAs transcribed from the plasmids (neither of which are translated in BTV infected cells), and the viral mRNAs (which are still translated) is the presence of a poly(A) tail in place of the viral 3'UTR.

This chapter reports the inhibition of 'poly-A' dependent translation as a result of BTV infection, while the translation of 'BTV-like' mRNAs is enhanced. The role of NS2 in BTV gene expression and translation was also explored and is discussed.

5.2: PCR template and plasmid production

The chloramphenicol acetyltransferase (CAT) open reading frame was amplified by PCR from the plasmid pGEM-CAT/EMCV/LUC, kindly provided by Dr. L. Chard, (Roberts et al., 1998), using KOD hotstart polymerase (Materials and Methods, section 2.8.2). PCR products were separated by agarose gel electrophoresis and purified from the gel using the illustra GFX PCR DNA and Gel band purification kit (Materials and Methods, sections 2.10 and 2.13).

The 5' and 3' untranslated regions (UTRs) of RSAvvvv/16 Seg-5 were added to the CAT open reading frame by PCR using the primers shown in Table 5.1. Three sequential PCR reactions were necessary to build up the full length UTRs, each time using the previous product as a template. A truncated T7 phage promoter was added to the 5' end, in such a way that BTV-like mRNA would be transcribed in an *in vitro* transcription reaction (Materials and Methods, section 2.22). The final PCR product, referred to as 5'/CAT/3', (Figure 5.1) was used as a template to generate further modified versions of the authentic 'segment'.

Primer Name	Primer sequence (5'-3')
BTV-16v 5'UTR+CAT	GTTAAAAAAAGTTCTCTAGTTGGCAACCACCAAAC ATGGAGAAAAAAA <u>TCACTGG</u>
BTV-16v Seg-5 3'UTR+CAT	gagtgctaagagaaaaatag ATAGAAAAGAATAAAAACAGAAATTAAACA <u>ATTA</u> <u>CGCCCCGCCCTGCCACTC</u>
BTV-16v Seg-5 3'UTR(2)	GTAAGTTGAAAAGTTCTAGTAGTAgagtgctaagagaaaaatag
BTV-16v Seg5 R	GTAAGTTGAAAAGTTCTAGTAGAGTG
SacII T7F	ATATCCGCGGTAAATACGACTCACTATA
SacII T7+CAT	TTAACCGCGGTAAATACGACTCACTATAGTTAA ATGGAGAAAAATCA <u>CTGG</u>
CAT + STOP R	<u>TTACGCCCCGCCCTGCCACTCATC</u>
CAT + STOP R+6nt	GTAAG TTACGCCCCGCCCTGCCAC
Seg-5 TR+EagI	GGCCGTAAGTTGAAAAGTTCTAGTAGAGTGC
SacII T7 CAT	TTAACCGCGGTAAATACGACTCACTATAGTTAA ATGGAGAAAAATC <u>ACTGG</u>
Seg-5 1757-1735R	TGAAAAGTTCTAGTAGAGTGCTAAGAG

Table 5. 1: The primers used to generate *in vitro* transcription templates.

Successive PCR reactions were performed to amplify the CAT open reading frame with the RSAvvvv/16 5' and 3' UTRs. The 3' primers were used to generate variations in the 3' terminus: BTV-16v Seg-5 R was used to generate 5'/CAT/3', CAT+STOP R+6nt was used to generate 5'/CAT/3'+6nt, Seg-5 TR+EagI was used to generate 5'/CAT/3'+EagI, CAT+STOP R was used to generate 5'/CAT and Seg-5 1757-1735R was used to generate 5'/CAT/3'-6nt. Underlined sequences target the CAT open reading frame (start and stop codons in bold). The lowercase sequence represents the overlapping sequence of the two primers used to generate the BTV 3' UTR in successive PCR reactions (PCR 2, Figure 5.1).

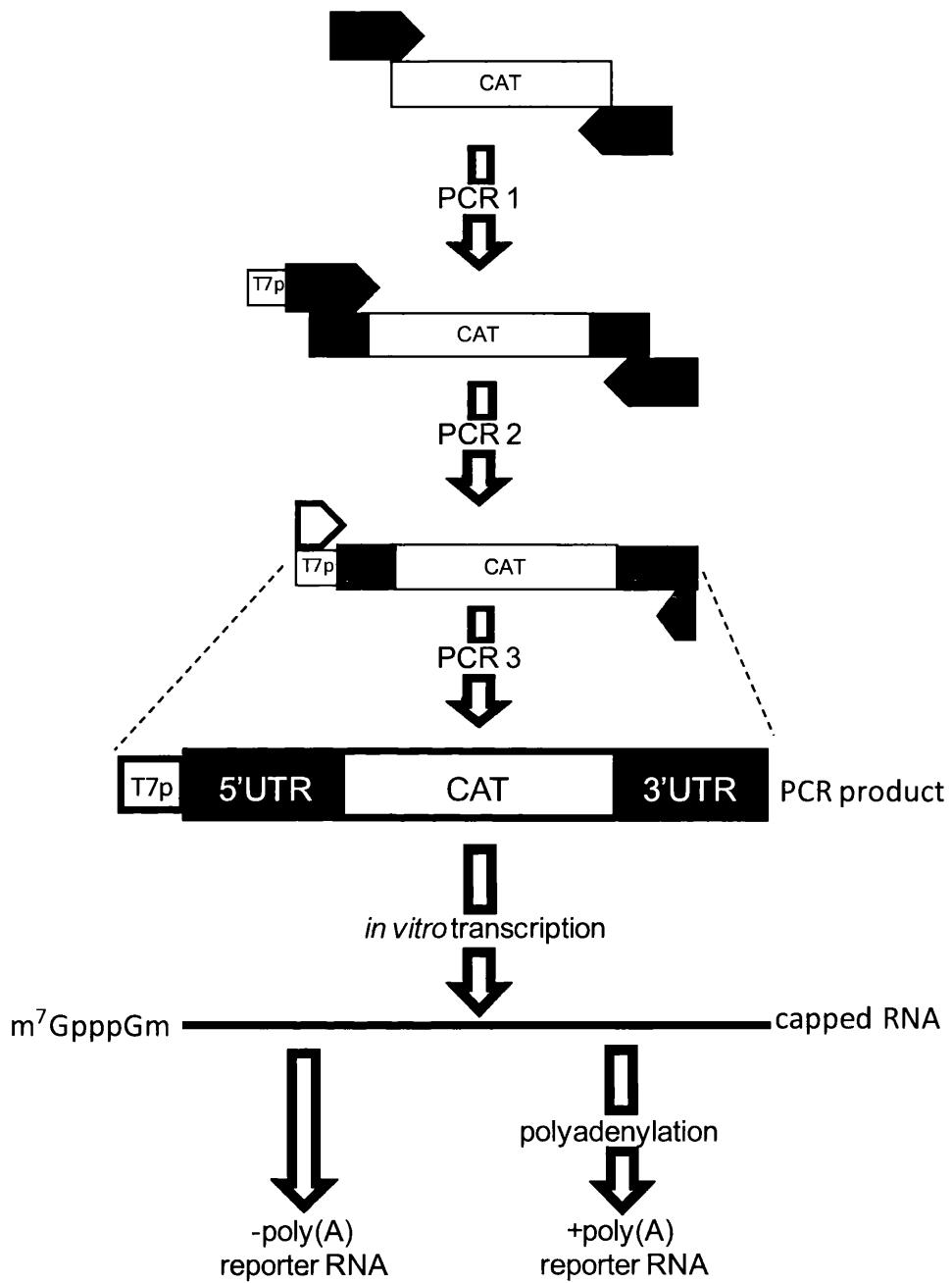


Figure 5. 1: *in vitro* synthesis of 'reporter' mRNAs.

A PCR product was generated for use as a template for *in vitro* transcription reactions. The CAT open reading frame was amplified using primers containing sections of the 5' and 3' UTR from BTV Seg-5 (which encodes NS1). The purified PCR product was used in subsequent rounds of PCR to include an upstream T7 promoter (T7p) as well as full-length BTV-UTRs, flanking the reporter gene. The final PCR product was used in an *in vitro* transcription reaction with anti-reverse cap analog (ARCA), to produce BTV-like mRNAs. The products of the *in vitro* transcription reactions were then either polyadenylated prior to being purified, (poly(A) RNA), or purified directly. These purified reporter RNAs were subsequently transfected into cells that were infected, or mock infected with BTV.

All PCR templates were sequenced using ABI BigDye chemistry using the primers in Table 5.2 to confirm the authenticity of the products.

Primer	Sequence
CAT 388-407F	AACCTGGCCTATTCCTAA
CAT 449-430R	CAGGGATTGGCTGAGACGAA

Table 5. 2: Primers used for the sequencing of CAT reporter construct PCR products.

5.3: Optimising the amount of RNA in transfection complexes

The amount of ssRNA generated using the coupled capping/transcription kit was sometimes limited. It was therefore considered important to conserve the amounts of RNA used in transfection experiments, wherever possible. However, it was also important that an adequate 'expression' signal was generated for detection by the CAT ELISA. The amount of RNA used per well for each transfection, was initially determined empirically by transfecting different amounts of the reporter RNA into uninfected BHK-21 cells in 24 well plates. The polyadenylated reporter mRNA was used, as this was expected to translate most efficiently in mammalian cells. The RNA was transfected in a final volume of 100 μ l using 0.25 μ l of Lipofectamine 2000 (Invitrogen) per 100 ng of RNA according to the manufacturer's conditions. The transfected cells were then incubated for a further 12 hours to allow translation, before CAT expression was detected by ELISA.

Transfections containing up to 50 ng of reporter mRNA/well failed to give an adequate positive signal by ELISA (Figure 5.2). However, when 100 ng of RNA was included in the transfection, a good level of signal was observed (Figure 5.2). Although further increases in the amount of transfected RNA did increase the level of signal obtained still further, it appeared to reach a plateau-level above 150ng. 100ng was therefore chosen for future experiments, as this not only gave a balance between signal and economy, it would also allow any significant inhibition or stimulation of translation to be detected. Although it was anticipated that 'BTV-like' reporter mRNAs (which do not have a poly(A) tail) would be translated only weakly in mammalian cells, even a 10-fold drop in efficiency would still give a sufficient CAT signal for detection.

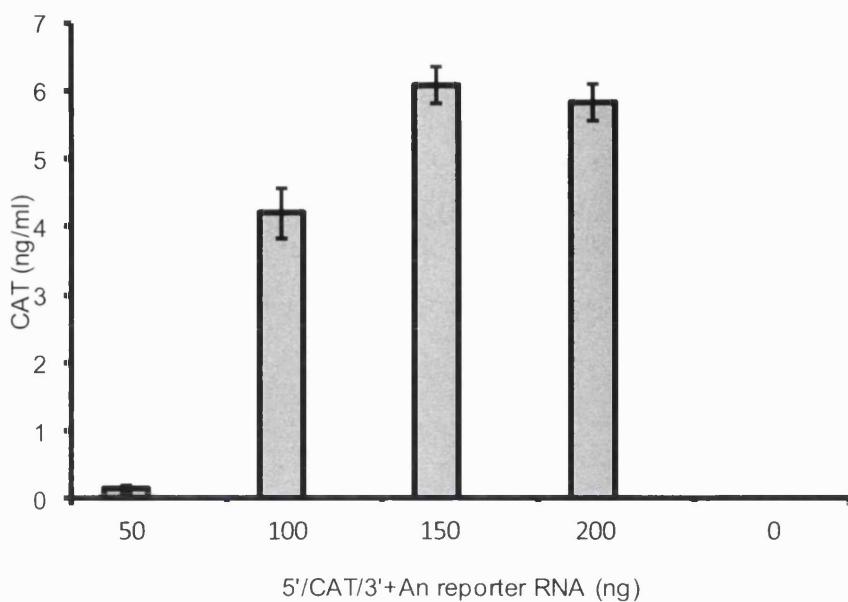


Figure 5. 2: Optimisation of the amount of reporter RNA used for transfection of BHK cells.

BHK-21 cells were transfected with 50, 100, 150 or 200 ng of *in vitro* transcribed and polyadenylated 5'/CAT/3' reporter RNA and assayed 12 hours later for CAT expression by ELISA. 50 ng of RNA gave very limited signal while 100 ng and above gave a good CAT signal. Error bars represent standard deviation, n = 3.

5.4: The effect of BTV infection on translation of 'reporter mRNAs'

BTV mRNAs have a 3' untranslated region (UTR), which varies in length and sequence between the different genome segments (Maan et al., 2008; Mertens and Sangar, 1985). The absence of a poly(A) tail makes it impossible for PABP to circularise the mRNA, and is therefore likely to reduce the relative efficiency of BTV translation in uninfected cells, compared to that of poly(A) tailed host-cell mRNAs (Wells et al., 1998).

The relative efficiency of BTV translation in mammalian cells was assessed by transfecting a number of different reporter mRNAs into uninfected, or BTV infected BHK-21 cells, at 8 hours post infection. A high level of infection was used in order to minimise background translation in transfected but uninfected cells (Figure 5.3). The different capped reporter mRNAs each contain the CAT ORF, with or without 5' and / or 3' UTRs derived from BTV-16 Seg-5. At 12 hours post transfection the cells were lysed and the level of CAT expression was determined by ELISA.

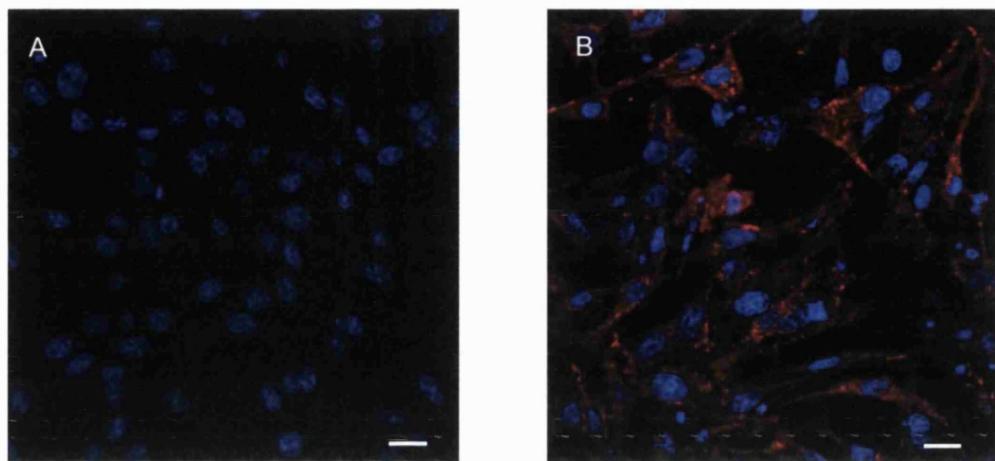


Figure 5. 3: Complete infection of BHK cell monolayers.

BHK cells were grown overnight and mock infected (A) or infected with the BTV-16 vaccine strain (B). At eight hours post infection the cells were fixed in 4% paraformaldehyde and immunostained using polyclonal antiserum against purified BTV cores to indicate infection, with Alexa-Fluor 568 secondary antibody labelling. Nuclei were labelled with DAPI. No labelling indicative of infection was observed in the mock infected cells, whereas there was evidence of infection in every cell of the BTV infected coverslip, implying background noise from uninfected cells would be minimal.
Scale bar = 10 μ m.

Reporter RNAs that contained neither the 3' or 5' UTR of BTV Seg-5 (i.e. simply capped RNA of the CAT ORF – indicated by 'CAT') failed to translate, regardless of the infection status of the cells. However, incorporation of the complete 5' and 3' UTRs of BTV Seg-5 into capped transcripts (indicated by the abbreviation '5'/CAT/3') did result in their translation in uninfected cells (Figure 5.4). This demonstrates that the BTV UTR(s) contain features which can direct translation of 'BTV-like' mRNAs. It also indicates that authentic viral mRNAs can be translated in the absence of additional viral factors. However, when 5'/CAT/3' reporter transcripts were introduced into cells that had also been infected with BTV, CAT expression from '5'/CAT/3' was significantly increased compared to that in uninfected cells (Ttest $P = 0.00051$). It therefore appears likely that viral or viral induced factors, generated during virus replication, can further enhance the translation of BTV mRNAs.

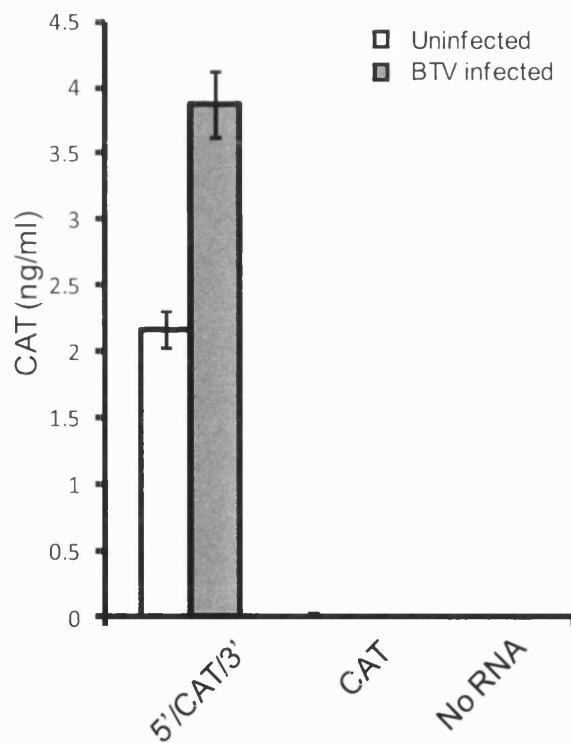


Figure 5. 4: BTV enhances the translation of BTV-like reporter mRNAs in BHK cells.

BHK-21 cells were transfected at eight hours post infection with 100 ng of *in vitro* transcribed reporter RNA and assayed for CAT by ELISA 12 hours later. A capped RNA representing only the CAT ORF (CAT), failed to translate regardless of the BTV infection status of the cells. However, reporter RNAs containing both BTV UTRs (5'/CAT/3') were translated in both uninfected (white bars) and infected (grey bars) cells, and BTV infection almost doubled the level of translation (Ttest $P = 0.00051$). Error bars represent standard deviation, $n = 3$.

Capped reporter transcripts lacking either the 5' UTR (indicated by the abbreviation CAT/3') or the 3'UTR (indicated by the abbreviation 5'/CAT) were only translated very inefficiently in either infected or uninfected cells (Figure 5.5) demonstrating the importance of both UTRs.

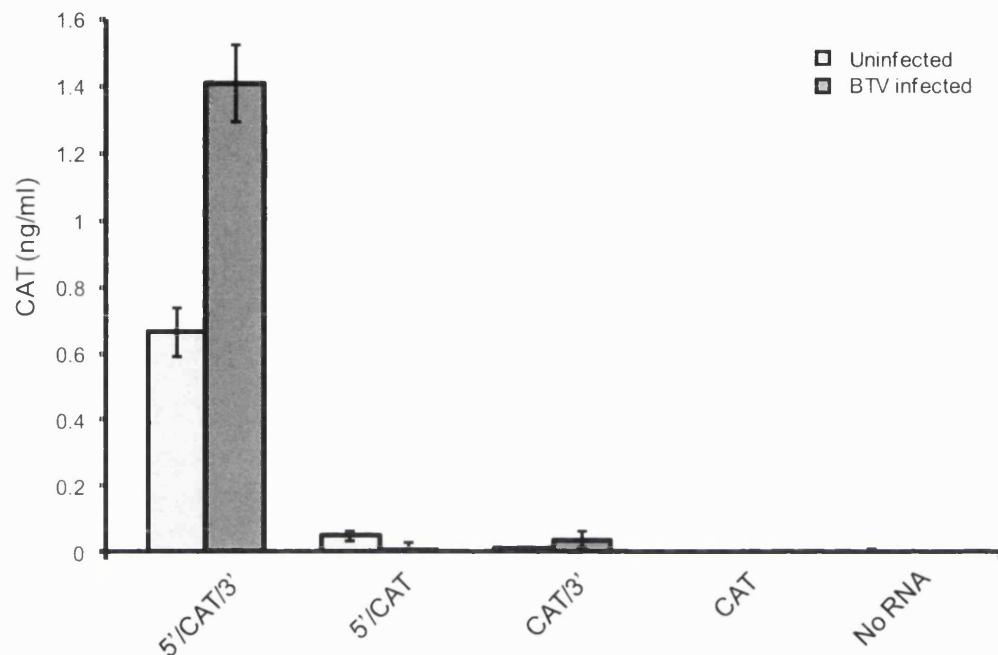


Figure 5. 5: Both UTRs are required for translation of BTV-like mRNAs in BHK cells.

BHK-21 cells were transfected at eight hours post infection, with 100 ng of *in vitro* transcribed reporter RNA and assayed for CAT by ELISA 12 hours later. BTV-like reporter transcripts with the complete 5' and the 3' UTRs of BTV segment 5 (5'/CAT/3') were translated in the absence of BTV when transfected into uninfected BHK-21 cells (white bars). Prior infection of the cells for 8 hours with BTV enhanced the observed translation (grey bars). When reporter transcripts devoid of either the 3' UTR (5'/CAT), and/or 5' UTR (CAT/3') were transfected in an identical manner as 5'/CAT/3', translation was minimal. Error bars represent standard deviation, n = 3.

5.4: Conserved terminal hexanucleotides are required for the translation of BTV mRNAs

The six nucleotides (3'CAUUAC) situated at the 3' terminus of each BTV mRNA, are highly conserved across the entire BTV species (Mertens and Diprose, 2004a; Rao et al., 1983). A reporter transcript was generated in which these six 3' terminal bases had been removed, but which was otherwise identical to 5'/CAT/3'.

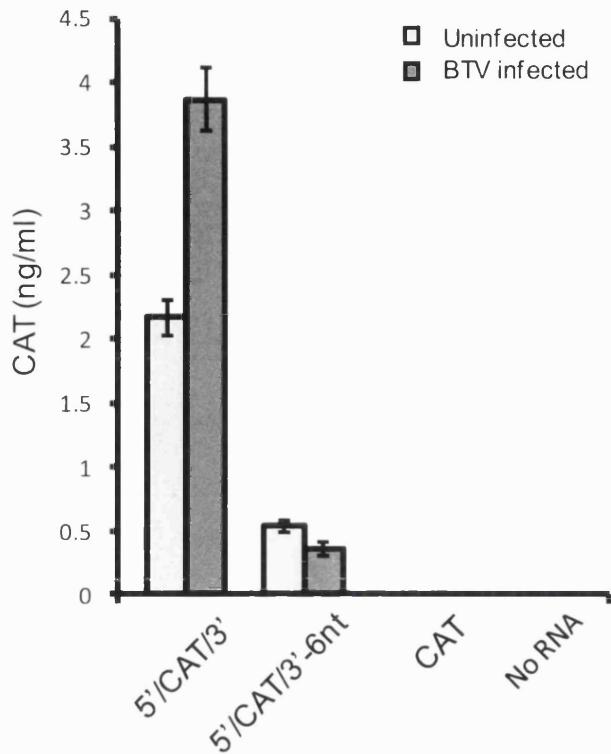


Figure 5. 6: The conserved terminal hexanucleotides of BTV mRNAs are necessary for efficient translation in BHK cells.

BHK-21 cells were transfected at eight hours post infection with 100 ng of *in vitro* transcribed reporter RNA and assayed for CAT by ELISA 12 hours later. In the absence of the BTV UTRs (CAT) no translation was observed. Translation of a reporter RNA with the 5' and 3' UTRs (5'/CAT/3') was more efficient when the cells had previously been infected with BTV (grey bars). In both infected (Ttest $P = 1.9 \times 10^{-5}$) and uninfected cells (white bars), the level of CAT detected by ELISA was dramatically reduced when the 3' terminal six nucleotides were removed (5'/CAT/3'-6nt). Error bars represent standard deviation, $n = 3$.

The absence of the conserved terminal hexanucleotide reduced translation of the reporter RNA in uninfected cells by four fold, and in infected cells and by greater than 10 fold (Ttest $P = 1.9 \times 10^{-5}$, Figure 5.6), demonstrating the importance of these bases for efficient translation of BTV mRNAs. However, the removal of most of the 3'UTR, except the terminal six nucleotides (5'/CAT/6nt, Figure 5.7), also abrogated translation, indicating that there are other critical features in the 3'UTR beyond the terminal six bases. The addition of extra nucleotides to the 3' terminus (5'/CAT/3'+EagI, Figure 5.7), also prevented translation, demonstrating that the 'context' of the terminal hexanucleotide is important, implying that they need to be positioned at the 3'end (Figure 5.7).

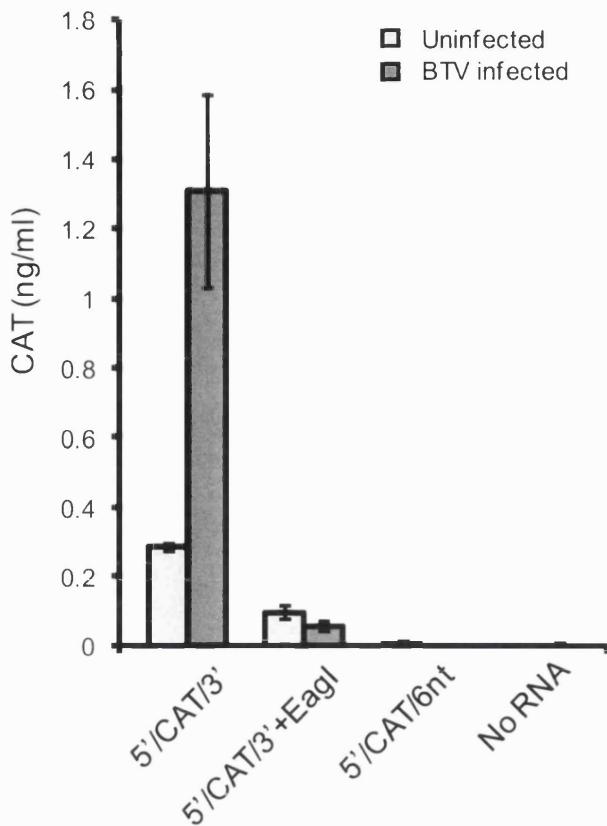


Figure 5. 7: The complete BTV 3' UTR and its position at the 3' terminus are important for translation in BHK cells.

BHK-21 cells were transfected at eight hours post infection with 100 ng of *in vitro* transcribed reporter RNA and assayed for CAT by ELISA 12 hours later. In addition to BTV-like (5'/CAT/3'), transcripts were made with additional bases at the 3' end (5'/CAT3'+EagI) or with the entire 3'UTR removed with the exception of the terminal six nucleotides (5'/CAT/6nt). Translation was observed for the BTV-like reporter transcripts, but the addition of nucleotides thus altering the 3' end, or removal of all but the terminal 6 nucleotides of the 3'UTR, abrogated translation in uninfected (white bars) or infected (grey bars) BHK-21 cells. Error bars represent standard deviation, n = 3.

5.5: The effect of BTV infection on poly-A dependent translation

It is well established that BTV infection induces shut-off of host-cell protein synthesis in certain mammalian cells (Huismans, 1970b; Mertens et al., 1984), yet it remains unclear at what point in the expression pathway protein synthesis is inhibited. Previous studies (Stirling, 1996) have suggested that, like orthoreovirus, there is a reduction in cap-dependent translation as infection proceeds (Skup and Millward, 1980; Stirling, 1996). However, the impact of BTV infection on poly(A) dependent translation (i.e. translation of polyadenylated host-cell relatively mRNAs), has not previously been examined.

Polyadenylated versions of the CAT reporter mRNAs described above (indicated by abbreviations 5'/CAT/3'+An, 5'/CAT+An, CAT/3'+An and CAT+An) were also transfected into uninfected, or BTV-infected BHK cells. In each case, the polyadenylated reporter mRNAs were translated more efficiently than the non-polyadenylated versions in uninfected cells. Indeed, polyadenylation of 5'/CAT/3' (to generate 5'/CAT/3'+An), enhanced CAT expression in uninfected cells by 14.6 fold (average values, Ttest p = 0.00051, Figure 5.8). The addition of a 3' poly(A) tail to 5'/CAT, CAT/3' or CAT also allowed relatively efficient translation in uninfected cells (Figure 5.8), unlike the non-polyadenylated versions of these RNAs where little or no translation was observed (Figure 5.5).

The four polyadenylated transcripts used in these experiments were translated with different efficiencies in uninfected cells (Figure 5.8). The 5'/CAT+An transcript, which has the BTV 5'UTR but lacks the 3'UTR, was translated most efficiently. The increased translation of this construct compared to that of the CAT ORF alone (CAT+An) suggests that the BTV 5' UTR has a positive influence upon translation in uninfected cells; an observation that was also supported by the greater level of translation detected with 5'/CAT/3'+An when compared to CAT/3'+An (which was the least translated reporter RNA). In contrast, (as demonstrated above) although the BTV Seg-5, 3'UTR is required for translation of the non-polyadenylated reporter RNA (5'/CAT/3'), it appeared to have a consistent inhibitory effect on translation of polyadenylated RNAs in uninfected cells (for example, compare expression 5'/CAT+An to 5'/CAT/3'+An, or CAT+An to CAT/3'+An in Figure 5.8).

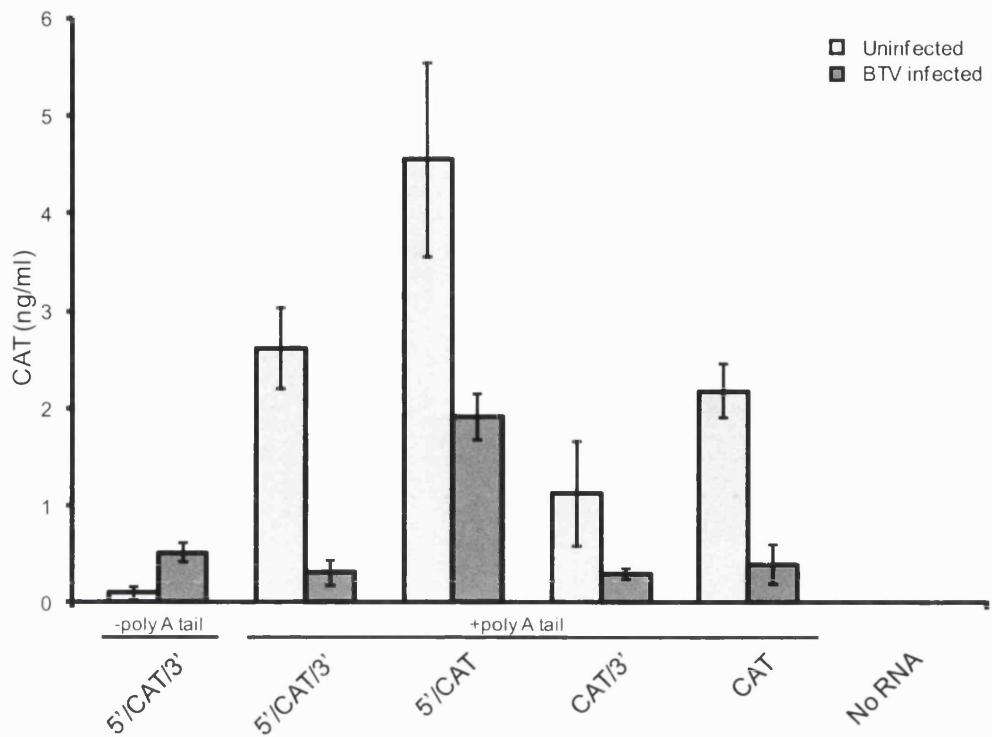


Figure 5. 8: BTV infection reduces translation of polyadenylated reporter mRNAs in BHK cells.

Polyadenylated reporter transcripts were used to investigate the effect of BTV on poly(A) dependent translation. Each transcript was transfected into uninfected (white bars) or infected (grey bars) BHK-21 cells. The addition of a poly(A) tail to 5'/CAT/3' greatly enhanced its translation in uninfected cells (Ttest $P = 0.00051$). In infected cells, the polyadenylated version of 5'/CAT/3' was translated to a level below the BTV-like non-polyadenylated (Ttest $P = 0.00081$). It was consistently observed that polyadenylated RNAs were translated to a lower level in infected cells compared to uninfected cells. The presence of the 3' UTR in polyadenylated transcripts appeared to have an inhibitory impact upon translation (compare 5'/CAT to 5'/CAT/3' and CAT to CAT/3'). Error bars represent standard deviation, $n = 3$.

BTV infection caused a dramatic drop in the translation of all of the polyadenylated reporter RNAs in BHK cells (by 41.66 to 84.75 %), including the reduction of 5'/CAT/3'+An translation to below that of the un-polyadenylated version (5'/CAT/3') in infected cells (Ttest $P = 0.00081$). Although there was also a reduction in the translation of 5'/CAT+An in infected cells, this was less dramatic than with the other transcripts, again suggesting that the presence of the 5' UTR exerts a positive influence upon translation in BTV infected cells.

5.6: The role of NS2 in translation of BTV mRNAs

Although the 3' and 5' UTRs are required for efficient translation of 'BTV-like' mRNAs, the increase in their translation efficiency after infection indicates that other BTV-specific factors are also involved in an 'expression enhancer mechanism'. Based on observations with rotavirus (RV) NSP3 it appears likely that one or more of the BTV NS proteins could be responsible for increasing the translation of BTV mRNAs (Piron et al., 1998; Vende et al., 2000). BTV NS2 can bind to viral ssRNA, although reportedly not to the conserved terminal bases of the segments (Huismans et al., 1987b; Theron and Nel, 1997). This ssRNA binding capability makes NS2 a strong candidate as a viral factor responsible for enhancing the translation of BTV mRNA.

A PCR product representing BTV Seg-8 (encoding NS2) was amplified from viral cDNA using primers targeting the terminal regions ('BTV-16v T7 S8F' [AATTTAATACGACTCACTATAGGGTTAAAAATCCTGAGTCATG] and 'BTV Seg-8R' [GTAAGTGTAAAATCCCCCCYAA]). These primers generated a PCR product that also has a T7 promoter situated at its upstream / 5' end, allowing it to be used as a template for *in vitro* transcription of positive sense RNAs (mRNAs) encoding NS2 (Materials and Methods, section 2.22.2). These mRNAs were polyadenylated (to allow them to be translated efficiently in the cell) and increasing amounts were co-transfected into uninfected cells, along with fixed amounts of the reporter 5'/CAT/3' (Figure 5.9).

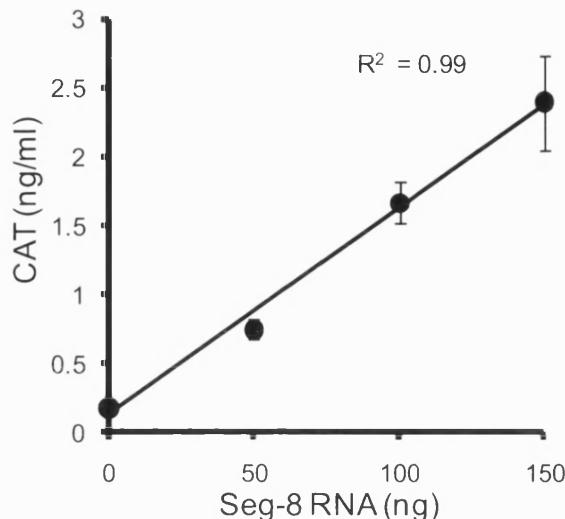


Figure 5.9: The effect of NS2 expression on translation of BTV-like reporter mRNAs.

BTV-like reporter mRNAs with complete 5' and 3' UTR (5'/CAT/3') were co-transfected into uninfected BHK-21 cells, along with increasing amounts of polyadenylated *in vitro* transcribed and polyadenylated, BTV Seg-8 mRNAs. The cells were lysed at 12 hours post transfection and the level of CAT measured by ELISA. Linear regression analysis was performed to assess the relationship between the amount of segment-8 RNA and the corresponding level of translation observed. Error bars represent standard deviation, $n = 3$.

Although the 5'/CAT/3' reporter RNA was only poorly translated in the absence of BTV infection (see above Figure 5.4), co-transfection with Seg-8 mRNA increased its expression levels. A positive correlation was also observed between the amount of Seg-8 mRNA transfected and the level of 5'/CAT/3' translation. Linear regression analysis indicated that this is a strong relationship with an R^2 value of 0.99 (Figure 5.9). These data support the hypothesis that NS2 is at least partially responsible for enhancing the translation of the BTV mRNAs.

5.7: The impact of NS2 on poly(A) dependent translation.

The data presented in section 5.6 implies that BTV NS2 may act as a functional homologue of RV NSP3. NS2 was shown to enhance the translation of BTV-like reporter RNAs in a manner reminiscent of NSP3. However NSP3 is also responsible for the shut-off of poly(A)-dependent translation by binding eIF4G in place of PABP (Piron et al., 1998). The displacement of PABP stops the translation initiation complex circularising polyadenylated RNAs, decreasing their efficiency of translation. To investigate whether NS2 was also responsible for inhibition of poly(A) dependent translation (as observed in section 5.5), increasing amounts of polyadenylated Seg-8 RNA were co-transfected into

uninfected BHK-21 cells, together with fixed amounts of either 5'/CAT/3' or 5'/CAT/3'+An. A positive correlation was again observed between the amount of co-transfected Seg-8 mRNA and the efficiency with which the BTV-like 5'/CAT/3' reporter RNA was translated (5'/CAT/3', Figure 5.10). It was also confirmed that the addition of a poly(A) tail greatly enhanced the translation of the reporter RNA in these uninfected cells (5'/CAT/3'+An, Figure 5.10). However, co-transfection of Seg-8 mRNA did not affect translation of the polyadenylated reporter RNA (Figure 5.9). These data indicate that although NS2 can stimulate translation of BTV like mRNAs, it is not responsible for the inhibition of poly(A) dependent translation observed during BTV infection. The host-translation shut-off process must therefore be mediated by a different mechanism to that suggested for rotavirus.

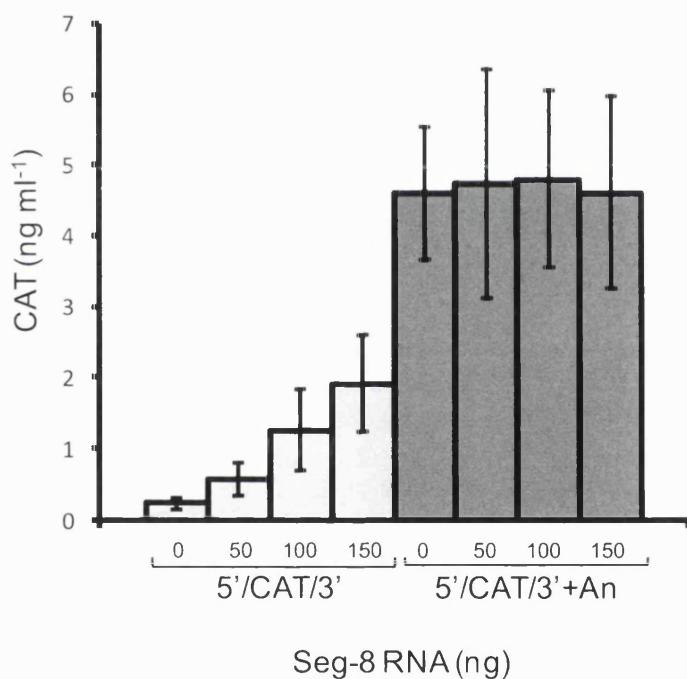


Figure 5. 10: NS2 does not appear to affect the translation of polyadenylated reporter RNAs.

BTV-like reporter mRNAs containing the complete 5' and 3' UTR, with or without a poly(A) tail (5'/CAT/3'+An and 5'/CAT/3' respectively) were co-transfected into uninfected BHK-21 cells, along with increasing amounts of polyadenylated *in vitro* transcribed and polyadenylated BTV Seg-8 mRNAs (to express NS2). The cells were lysed at 12 hours post transfection and the level of CAT measured by ELISA. A positive correlation was observed between the amount of Seg-8 mRNA and translation of the BTV-like reporter RNA (5'/CAT/3'). However, no effect was observed on expression from 5'/CAT/3'+An. Error bars represent standard deviation, n = 3.

5.8: Discussion

BTV infection of most mammalian cells causes a severe cytopathic effect (CPE), culminating (in BHK-21 cells) in lysis and death within 24-48 hours post infection. An important feature of this CPE is shut-off of host cell protein synthesis, so that the majority of proteins that are synthesised in BTV infected mammalian cells post-shut-off, are virus derived. A control mechanism must therefore exist which allows BTV mRNAs (which are capped but non-polyadenylated) to be expressed, while translation of mammalian host-cell mRNAs is inhibited.

In addition to RNA-protection and stabilising functions (Ross, 1995), both poly(A) tails and 3' UTRs can fulfil important roles in translation. The poly(A) tail of host-cell mRNAs binds to the poly A binding protein (PABP), which can also bind to eIF4G (Cheng and Gallie, 2007; Imataka et al., 1998; Le et al., 1997). eIF4G also interacts with eIF4E, which binds to the Cap 1 structure at the 5'end of the mRNA, thereby forming a closed loop/circle (Wells et al., 1998). This circularisation of mRNAs is thought to be important for their efficient translation, providing a stable initiation complex, and promoting ribosome recruitment, initiation of translation and ribosome recycling. The UTRs of non-polyadenylated mRNAs have also been shown to contain numerous features, that include both translation enhancer and suppressor functions (Li and Brinton, 2001).

By cleaving, or otherwise eliminating proteins that are essential for the translation of host-cell mRNAs, expression can be biased towards viral proteins. Foot and mouth disease virus (FMDV) proteins L^{pro} and 3C induce cleavage of eIF4G and eIF4A, while the HIV protease and the calicivirus '3C-like' protease cleave PABP (Alvarez et al., 2006; Belsham et al., 2000; Devaney et al., 1988; Kuyumcu-Martinez et al., 2004; Kuyumcu-Martinez et al., 2002). If initiation of host cell translation is disrupted, the virus must use an alternative mechanism to ensure the translation of its own proteins. For example the picornaviruses and flaviviruses initiate 'cap-independent' translation using an internal ribosomal entry site (IRES) (Belsham, 2009).

The translation mechanisms used by the rotaviruses (RV, a distinct genus within the family *Reoviridae*) have been more thoroughly explored than those used by BTV and the other orbiviruses. The RV NSP3 protein binds eIF4G in place of PABP and circularises the viral mRNA by interacting with the terminal four bases at its conserved 3' end (Deo et al., 2002; Groft and Burley, 2002). NSP3 binding to eIF4G also evicts PABP, shutting-off poly(A) dependent translation (Piron et al., 1998; Poncet et al., 1993).

However, RV NSP3 may not be essential for translation (Montero et al., 2006) and the non-polyadenylated 3' UTR of Dengue virus can itself bind to PABP (Polacek et al., 2009).

Although there is a generalised inhibition of macromolecule synthesis in mammalian cells that are infected with BTV (Huismans, 1970a; Huismans, 1970b), little is known concerning the mechanisms involved, or the way in which BTV protein synthesis is maintained. Although BTV mRNAs are capable of being translated in uninfected cells (in the absence of viral gene expression) viral protein synthesis is relatively inefficient, compared to that of host-proteins translated from polyadenylated mRNAs. However infection causes modifications within the host cell that increase the relative efficiency of translation of the majority of BTV mRNAs (Figure 5.11). The lack of translation of 'BTV-like' mRNAs in either infected or uninfected cells, after the UTRs have been removed (Figure 5.11), indicates that these sequences perform a significant functional role in translation, possibly influencing the efficiency of initiation.

It has been suggested that inverted repeats in the 5' and 3' UTRs of BTV mRNAs (Roy, 1989) interact, increasing the efficiency of translation (Markotter et al., 2004). Indeed, removal of either the 5' or the 3' BTV UTR abolishes translation, confirming that both BTV UTRs are required. In contrast, the 3' UTR alone of Bunyamwera virus (another segmented arthropod borne virus which inhibits poly(A) dependent host-cell translation) is necessary and sufficient for efficient translation of the viral mRNAs (Blakqori et al., 2009). Terminal repeats in the RV mRNAs are also required for minus strand synthesis and genome replication (Chen et al., 2001; Chen and Patton, 1998; Patton et al., 1996).

	Infected	Uninfected
5'/CAT/3'	++	+
5'/CAT'	-	-
CAT/3'	-	-
5'/CAT/3'+EagI	-	-
5'/CAT/3'-6nt	-	-
5'/CAT'+6nt	-	-
CAT	-	-
5'/CAT/3'+A(n)	++	+++

Figure 5.11: A summary of the translation efficiencies of the RNA reporter constructs in the presence or absence of BTV infection.

Capped reporter RNAs were transcribed *in vitro* from PCR products representing the CAT open reading frame, flanked by variations of the RSAvvvv/16 Seg-5 untranslated regions. 5'/CAT/3' represents the complete 5' and 3' UTRs and is translated in both infected and uninfected cells, although infection stimulates the level of translation of this RNA. Removal of the 3' UTR (5'/CAT/) or 5' UTR (CAT/3') abrogated translation, as did the addition of additional bases to the 3' end (5'/CAT/3'+EagI), removal of the terminal hexanucleotide (5'/CAT/3'-6nt) or removal of the 3' UTR with the exception of the terminal hexanucleotide (5'/CAT'+6nt). Polyadenylation of 5'/CAT/3' dramatically enhanced its translation in uninfected cells, whilst the level of translation was reduced in infected cells. Reporter RNAs were transfected into uninfected BHK cells or cells infected with RSAvvvv/16. The level of CAT translated was measured by ELISA 12 h post-transfection.

RV mRNAs terminate in a conserved sequence (5'....GACC3') which interacts with NSP3, in a manner analogous to the poly(A) tail of eukaryotic mRNAs interacting with PABP. Removal of the conserved 3' terminal hexanucleotide from BTV mRNAs abolished translation, demonstrating that these terminal residues are also critical for BTV protein synthesis. Adding nucleotides to the 3'end of the mRNA (so that the hexanucleotide was no longer at the terminus) also interfered with translation. The

requirement for the terminal sequence to be present and ‘exposed/situated’ at the terminus is consistent with the proposed model for the RV translation, where the 3’ terminal cytosine interacts with NSP3 (Varani and Allain, 2002). However, removal of the remainder of the BTV 3’ UTR, apart from the terminal hexanucleotide, did not allow efficient translation, demonstrating that other aspects within the 3’ UTR (possibly inverted repeats) are also required.

The level of translation from polyadenylated BTV-like transcripts, was reduced to a level below that of unpolyadenylated mRNAs in BTV infected cells, confirming that BTV can influence mammalian protein expression sufficiently to bias translation towards viral proteins, a scenario which is clearly advantageous to virus replication.

Although the 3’ UTR would not normally be followed by a poly(A) tail, the presence of the BTV Seg-5 - 3’ UTR within a polyadenylated transcript, reduced the level of translation to below that of a polyadenylated transcript without either BTV UTR, regardless of the infection status of the cells. This suggests that the sequence of the 3’ terminal region interacts with components of the host cell translation apparatus, although the mechanism involved is unknown.

Data obtained and reported here for BTV indicates similarities with the RV translation mechanism, suggesting that one of the BTV proteins may be a functional homologue for RV- NSP3. The NS2 protein of BTV binds ssRNA, and could play a similar role to NSP3 in the translation of viral mRNAs. In co-transfection studies, the level of expression from the BTV-like reporter RNA, showed a linear correlation with the amount of co-transfected Seg-8 mRNA (encoding NS2). This indicates that BTV NS2 is indeed involved in the control of BTV translation. Although these data are consistent with NS2 performing a comparable role to RV-NSP3, previous studies have indicated that, unlike NSP3, NS2 does not directly interact with the conserved 3’ hexanucleotide (Theron and Nel, 1997).

To investigate further the hypothesis that NS2 acts as a functional homologue of RV NSP3, the impact of NS2 upon the translation of polyadenylated reporter RNAs was investigated. Interestingly, the co-transfection of increasing amounts of Seg-8 mRNA encoding NS2 did not decrease the efficiency of 5’/CAT/3’+An translation, as would be expected if NS2 was performing in an identical fashion to RV NSP3. It remains possible that the increased efficiency of the poly(A) dependent translation of the reporter 5’/CAT/3’+An masks any inhibitory effect of NS2. However, a clear response was observed with the unpolyadenylated 5’/CAT/3’.

These data indicate that, whilst NS2 enhances the translation of viral mRNAs, it does not perform all of the functions associated with NSP3. There must therefore be an alternative process by which BTV represses the translation of polyadenylated mRNAs. This result is not necessarily surprising in light of the different biological processes involved in the replication cycle of BTV compared to RV. The protein synthesis shut-off mechanism of BTV must only occur in mammalian cells whilst insect cells remain largely unaffected. It can be hypothesised that a finer level of control is required to achieve such a level of specificity, possibly with the involvement of cap-dependent translational shut-off (Skup and Millward, 1980; Stirling, 1996). Further work is required in order to more precisely elucidate the mechanism by which poly(A) dependent translation is inhibited.

The work described here uses BTV Seg-5 as a paradigm for the other BTV genome segments. Although BTV NS3 proteins can be generated in large amounts in infected insect cells (Guirakhoo et al., 1995), they are not easily detected in BTV infected mammalian cells. This suggests that unlike the other genome segments, Seg-10 mRNAs are not translated efficiently in mammalian systems even after host-cell shut-off. The BTV UTRs vary in length and sequence between the different genome segments, suggesting that these sequences could also control variations in expression levels between the different viral genes in these different systems. In particular the BTV NS3/NS3A proteins are very poorly expressed in mammalian cells, although Seg-10 mRNA is synthesised abundantly by the virus core and is translated efficiently in insect cells (Guirakhoo et al., 1995). The level of expression of particular BTV genes (e.g. those expressing NS1 and NS3/3A – Seg-5 and 10 respectively) could influence the development of CPE and virus pathogenicity (Guirakhoo et al., 1995; Owens et al., 2004). Variation in the mRNA UTRs may therefore influence the level of pathogenicity of different viruses in different hosts.

Further work is required to precisely define the role of different regions of the BTV mRNAs in translation, both for Seg-5, Seg-10 and the other segments. A similar approach could be used to study BTV translation in insect cells and determine quantitative differences in the level of translation of particular genes, in particular Seg-10.

Chapter 6: Investigating the impact of BTV infection upon mitosis

6.1: Introduction

Cells cultured or infected in the absence of serum divide slowly if at all. It was therefore thought to be more physiologically relevant if serum was included in the culture medium for experiments concerning BTV replication in cells. The presence of serum would allow cells to divide and respond to viral infection. The ability for a cell to exist in a resting state without dividing allows it to survive periods when nutrients are limited or absent. This may, however, also restrict its ability to respond to viral infection.

The cell cycle itself can be divided into multiple stages. The most basic division of the cell cycle is into interphase and mitosis. The interphase period can be further subdivided into the G₁, S and G₂ phases. After mitosis and cytokinesis the cell enters the 'G₀' or 'G₁' phase (G = gap). Cells in G₀ are often regarded as 'quiescent' or 'resting' and may either die or re-enter the cell cycle (G₁). The G₁ phase is largely a period of growth during which the cell prepares for duplication of the cellular DNA. The S (synthesis) phase follows G₁ and is the period in which the cellular DNA and the centrosome are duplicated. After S phase the cell enters the G₂ phase where the cell prepares for the cell division stage: mitosis.

Mitosis can also be divided into a series of steps: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. During prophase the chromatin condenses into chromosomes and a microtubule spindle forms. The microtubules are anchored to the centrosomes which by this point have moved to opposite poles of the cell. Prophase is followed by prometaphase during which the nuclear envelope breaks down and the microtubules polymerise through the cell towards the opposite pole in order to attach to the chromosomes via the kinetochores. Metaphase is a period of organisation for the chromosomes and the microtubule spindle. The chromosomes align across a metaphase plate whereby each chromosome is attached to both poles with equal force. The cell does not progress to the next stage, anaphase, until all of the chromosomes are correctly attached and aligned. During anaphase the two chromatids separate and each is pulled towards its respective pole by shortening of the microtubules that are attached to the kinetochores. The last stage of mitosis is telophase. During telophase the cell is stretched and the nuclear membrane re-forms around the chromatids. The cell is divided into two daughter cells in a final step known as cytokinesis.

In order to maintain the integrity of successive cell karyotypes, the cell cycle, and in particular mitosis, has evolved to be exquisitely sensitive to even slight aberrations from the ideal (Rieder and Maiato, 2004).

There are many examples of viruses altering the cell cycle at different stages in order to favour their replication, e.g. at the G1/S boundary or the G2/M boundary. Although cell cycle arrest may be beneficial to the virus, some of the mechanisms by which viruses modulate the cell cycle remain unclear.

Examples of viruses that modulate the cell cycle include human parvovirus B19, a small DNA virus which induces cell cycle arrest at the G2/M phase by inhibiting the nuclear import of cyclin B1 (Morita et al., 2001). Human cytomegalovirus (CMV) also blocks cells entering S phase from G1, thus biasing the cellular environment towards viral replication (Dittmer and Mocarski, 1997). There are also reports of insect viruses causing cell cycle arrest. *Autographa californica* nucleopolyhedrovirus (AcMNPV) is a large DNA virus of the family *Baculoviridae* which arrests Sf9 insect cells in the G2/M phase (Braunagel et al., 1998). Similarly, *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus (HaSNPV), causes a G2/M phase arrest in Hz-AM1 cells due to an accumulation of cyclin B1, in a manner reminiscent of parvoviruses (Zhou et al., 2004).

There are very few reports of cell cycle modulation within the *Reoviridae*. The T3D and T3A strains of orthoreovirus have both been shown to inhibit cellular proliferation by inducing a G2/M phase block via the viral attachment protein sigma1 (Poggioli et al., 2000).

Insect cells e.g. KC or C6/36 infected with BTV do not show overt cytopathic effect (CPE), despite supporting a persistent infection. It can be assumed therefore that BTV does not induce a lethal cell cycle arrest in these insect cells. However, severe CPE are observed in mammalian cells e.g. BHK-21 cells, although the precise molecular mechanism(s) involved are not fully understood. The BTV protein NS3/3A has been implicated in permeabilising the cell membrane and therefore in cell destruction (Han and Harty, 2004). However, the shut-off of host cell protein synthesis observed in BTV infected mammalian cells (see chapters 4 and 5) is also considered likely to result in cell death and BTV has been shown to induce both the intrinsic and extrinsic apoptosis pathways (Mortola et al., 2004; Nagaleekar et al., 2007).

In one study by Hu *et al* (2008) it was shown that BTV is cytotoxic to human cancer cells and prior to cell death, the cells were arrested in the sub-G1 phase of cell division. These data indicate that BTV has a significant effect on the cell cycle and that

cell division may be an important aspect in the BTV lifecycle (and cytopathogenesis) in mammalian cells.

This chapter describes results concerning the effect of BTV infection on mitosis in mammalian cells.

6.2: The impact of serum

The number of BHK cells in the mitotic phase of the cell cycle was determined for cells growing in the presence or absence of 10 % (v/v) foetal bovine serum (FBS). Mitotic cells were identified by the presence of condensed chromosomes, as revealed by DAPI staining. The number of mitotic cells was counted by confocal microscopy in 25 randomly selected fields of infected or uninfected cultures, with an average of 1.6 cells with 'condensed' nuclei per field in uninfected cultures, but 18.76 in infected cultures (Figure 6.1). Although a high level of variation was observed in the infected cultures with a standard deviation of 12.98, this difference was highly significant as determined by Ttest ($p = 1 \times 10^{-6}$, Figure 6.1).

In contrast, < 10 % of cells were mitotic in cell cultures grown in the absence or FBS, regardless of the infection status (not shown).

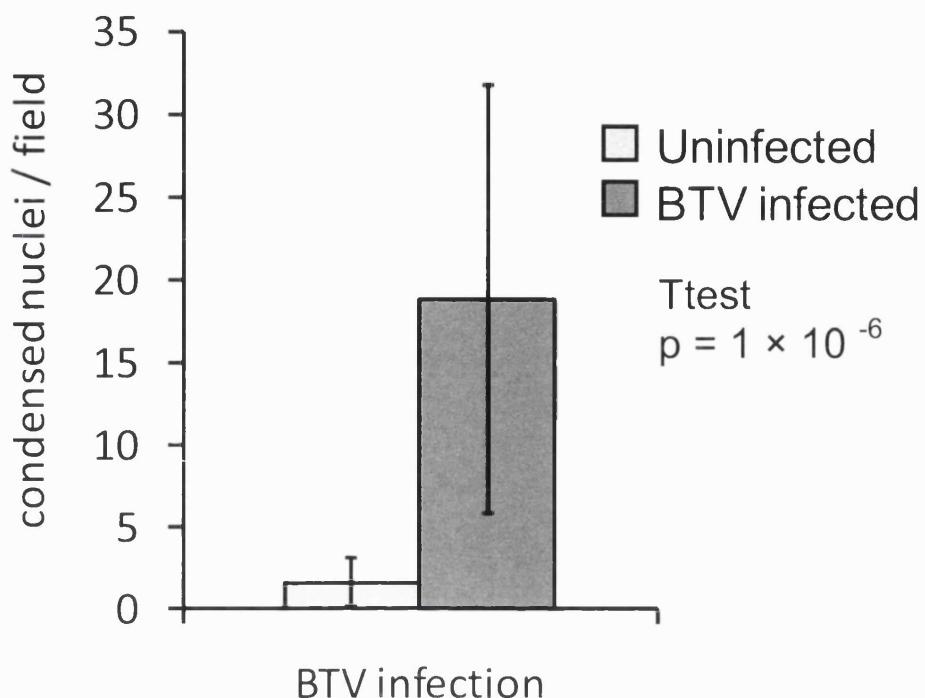


Figure 6. 1: The number of cells in mitosis in infected and uninfected cells.

BHK-21 cells were plated on glass coverslips and cultured overnight to approximately 70 % confluence. The cells were either 'mock infected' or infected with 300 μ l of a 10 % (v/v) dilution of RSAvvvv/16 cell culture supernatant for 30 minutes at room temperature. The inoculum was discarded and replaced with 1 ml of medium containing 0 or 10 % foetal bovine serum (FBS), and the cells were incubated at 37 °C for 24 hours. The cells were fixed in 4 % paraformaldehyde, and immuno-labelled using an antibody to NS2 (Orab 1, Materials and Methods, Table 2.10). The number of cells containing condensed chromosomes was counted in randomly selected fields of view in the confocal microscope. A significantly higher number of cells with condensed nuclei were observed in cells infected with BTV compared to uninfected cells.

The most conspicuous feature of the infected cultures examined by confocal microscopy, was the number of rounded cells, apparently locked in mitosis. The intracellular distribution of the chromosomes in these cells was also different from that expected for any of the 'normal' mitotic phases. Immunolabelling of NS2 showed that it co-localised with the condensed chromosomes in infected cells.

To investigate these phenomenon further, infected and uninfected cells were cultured in the presence of 10 % FBS and fixed at 16 hours post infection then immunolabelled for NS2 and α -tubulin (which labels microtubules/the mitotic spindle). The uninfected cells had the expected pattern of α -tubulin distribution, with mitotic cells

containing a mitotic spindle and a 'metaphase plate' (Figure 6.2). In contrast immunolabelling of BTV infected cells revealed a disorganised pattern of α -tubulin distribution, often with several mitotic spindles and condensed chromosomes that were disorganised and obviously unattached to a mitotic spindle (Figure 6.2). The BTV NS2 protein was again observed in association with condensed chromosomes (Figure 6.2D).

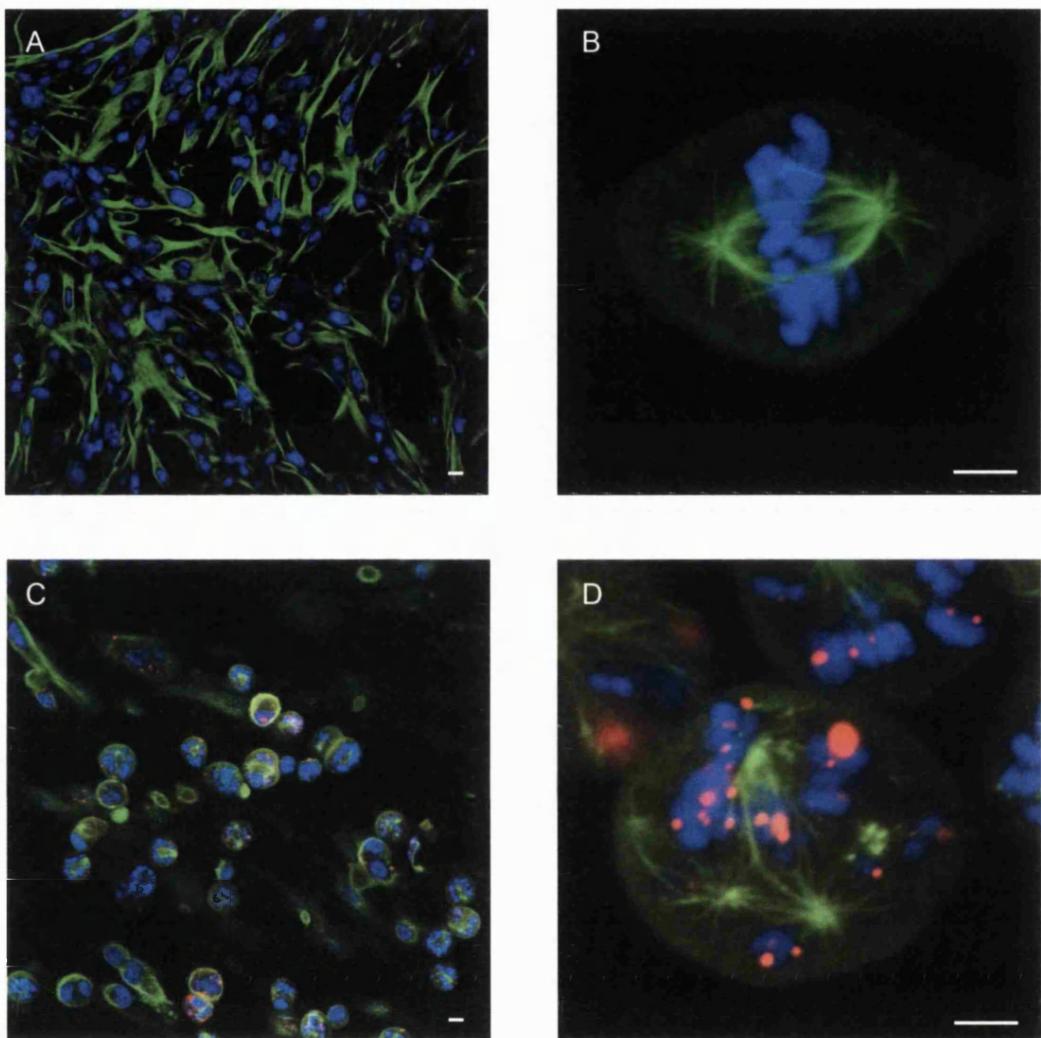


Figure 6.2: BTV infection induces BHK-21 cell arrest in mitosis.

BHK-21 cells were plated on glass coverslips. The cells were mock infected or infected with RSAvvvv/16 and incubated in the presence of 10% FBS. The cells were fixed at 16 hours post infection and immunolabelled using antibodies to NS2 and α Tubulin (labelling the microtubules). The nuclei/chromosomes were stained using DAPI. The uninfected cell culture contained very few mitotic cells (A) compared to the infected culture where rounded, mitotic cells were abundant (C). The mitotic events in uninfected cells appeared normal and matched the expected patterns e.g. the chromosomes (blue) arranging at metaphase plate (B). The mitotic cells infected with BTV contained a disorganized arrangement of condensed chromosomes, multiple microtubule spindles (green) and an association of NS2 (red) labelling with the condensed chromosomes (D).

Scale bar = 10 μ m.

6.3: Other viruses and other cells

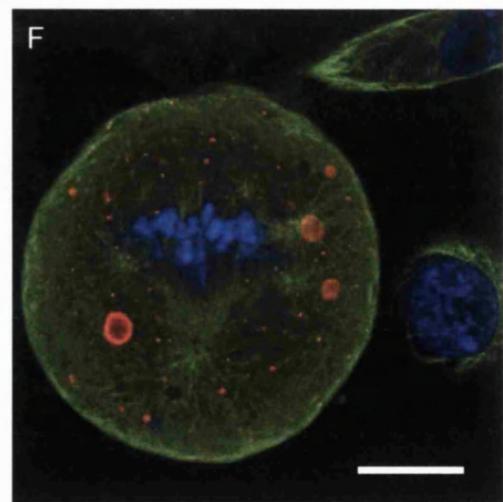
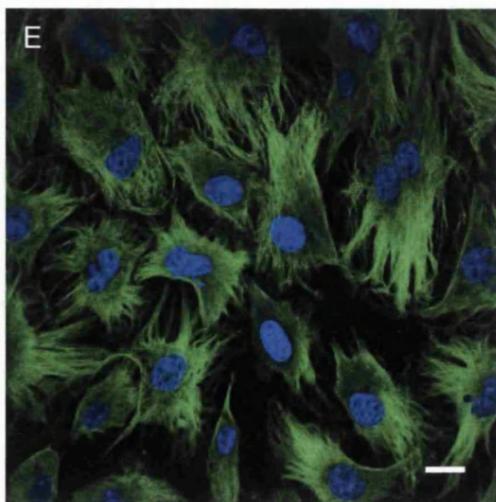
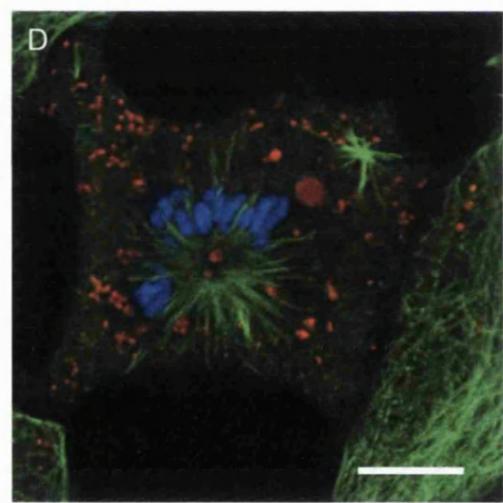
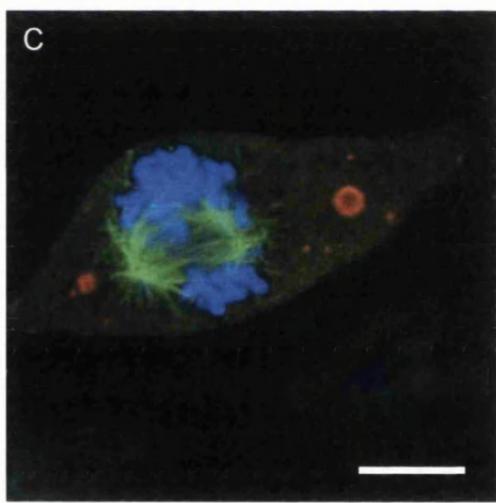
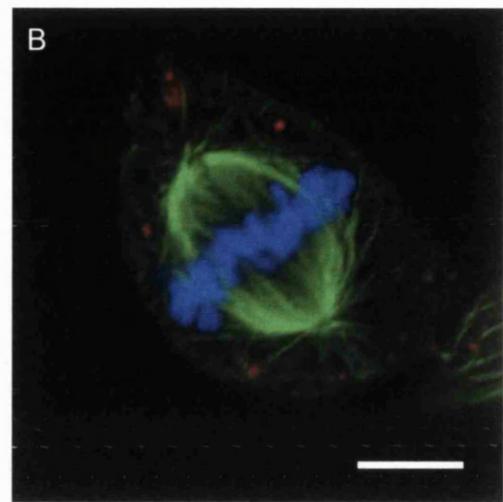
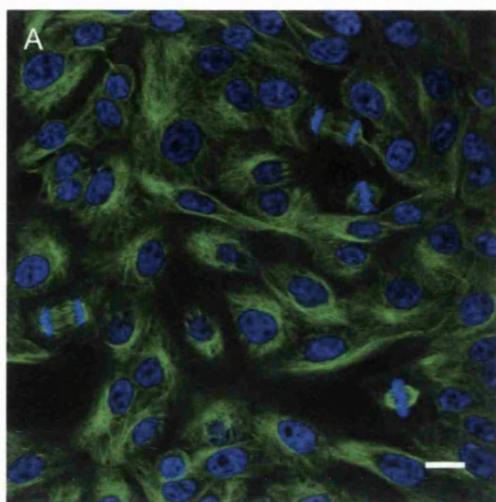
The arrest of mitosis by BTV infection was initially observed using the RSAvvvv/16 strain, which is highly adapted to BHK-21 cells. It may therefore have acquired particular characteristics for replication in these cells. There is evidence in the literature that the cytopathology induced by BTV varies according to cell type (DeMaula et al., 2001; Wechsler and McHolland, 1988). Vero cells and bovine pulmonary arterial endothelial (BPAEC) cells were therefore also tested to see if other cells were affected in a similar manner. Uninfected Vero and BPAEC cells showed 'normal' mitotic processes and spindle development (Figure 6.3A). Although some of the infected Vero cells showed a normal mitotic pattern of chromosomes (Figure 6.3B), there were also infected cells with multiple spindles, and condensed chromosomes that were arranged parallel to rather than at right angles across the microtubule spindle (as previously observed in BHK-21 cells - Figures 6.3C and 6.3D). Similar results were also obtained in infected BPAEC cells, with evidence of multiple microtubule spindles and apparently random accumulations of condensed chromosomes (Figure 6.3E and 6.3F).

Figure on following page.

Figure 6. 3: Aberrent mitosis is induced by BTV in multiple cell lines.

Vero (A-D) or BPAEC (E-F) cells were plated on glass coverslips. The cells were mock infected (A and E) or infected (B, C, D and F) with RSAvvvv/16 and incubated in the presence of 10% FBS. The cells were fixed at 16 hours post infection and immunolabelled using antibodies to NS2 and α Tubulin (labelling the microtubules). The nuclei/chromosomes were stained using DAPI (blue). Uninfected vero and BPAEC cells (A and E) demonstrated normal mitotic events with normally distributed microtubules (green). Some of the mitotic events in infected vero cells (containing NS2, red) appeared normal (B). However, there were examples of aberrant mitosis in both vero and BPAEC cells with incorrectly assembled spindles and a disorganised arrangement of chromosomes.

Scale bar = 10 μ m



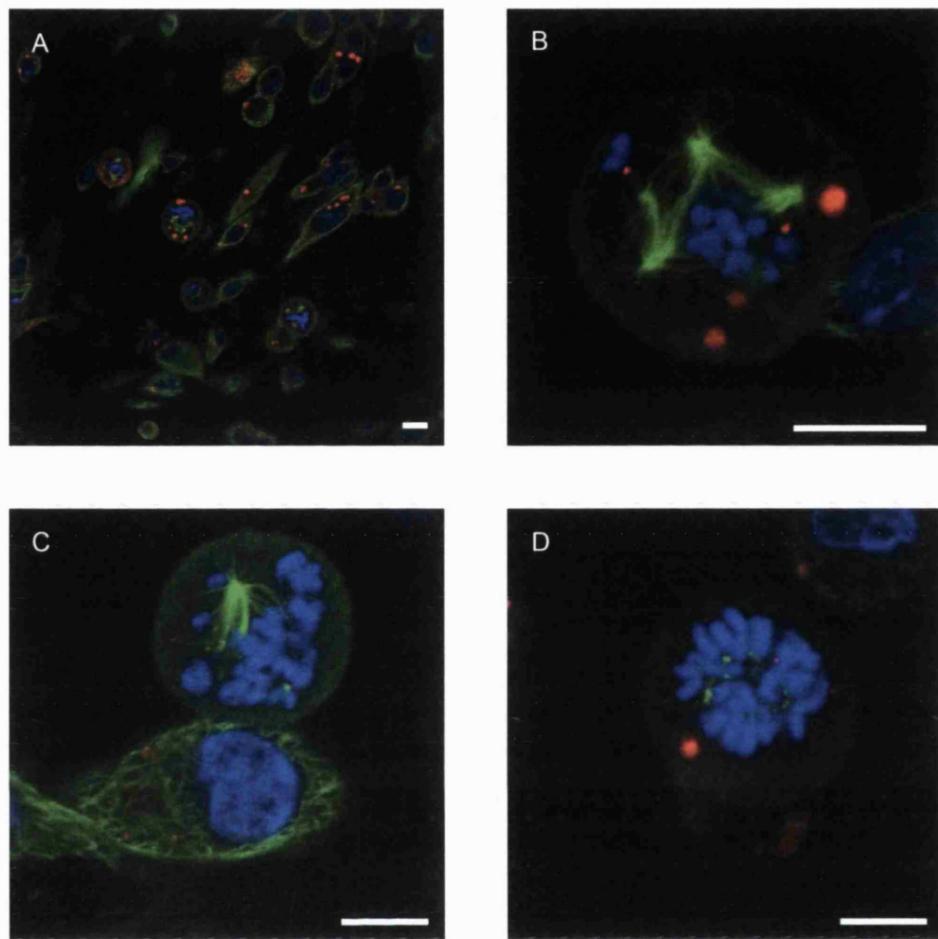


Figure 6.4: Aberrant mitosis is induced by different strains of BTV.

BHK-21 cells were plated on glass coverslips. The cells were mock infected or infected with the BTV-1 reference strain (RSArrrr/01, A and B) or the UK BTV-8 field strain (UKG2008/34, C and D) and incubated in the presence of 10% FBS. The cells were fixed at 24 hours post infection and immunolabelled using antibodies to NS2 and α Tubulin (labelling the microtubules). Nuclei/chromosomes were stained using DAPI. The chromosomes (blue) of mitotic cells were randomly distributed throughout the cell with multiple and/or disorganized microtubule spindles (green) evident. The NS2 (red) protein was also found associated with the condensed chromosomes. Scale bar = 10 μ m.

These results demonstrate that several cell lines were susceptible to the mitosis-blocking mechanism by BTV, although BHK-21 cells appeared to be the most susceptible.

BHK-21 cells infected with the reference strain of BTV-1 (RSArrrr/01) or a UK field strain of BTV-8 (UKG2008/34), also showed evidence of abnormal cell divisions, with multiple spindles and NS2 associated with the chromosomes (Figure 6.4). This indicates that these phenomena are not specific to RSAvvvv/16 and are not simply the result of multiple passage and adaptation to cell cultures.

6.4: The Microtubule organising centre (MTOC)

A conspicuous feature of the aberrant mitotic cells detected in BTV infected cell cultures, was the presence of multiple and asymmetrical microtubule spindles. To determine whether each microtubule spindle contained a microtubule organising centre (MTOC), BHK-21 cells were infected (or mock infected) with RSAvvvv/16. At 24 hours post infection the cells were fixed and immunolabelled for α -tubulin (microtubules) and γ -tubulin (a component of the MTOC). ‘Normal’ uninfected cells contained an irregular network of α -tubulin in non-mitotic cells, but a highly organised microtubule spindle in cells undergoing mitosis (Figure 6.5). In uninfected but non-mitotic cells, γ -tubulin was organised as a single perinuclear dot (Figure 6.5A). However, in cells undergoing mitosis, the γ -tubulin was located at the poles of each microtubule spindle, confirming its location at the MTOC (Figure 6.5B-D).

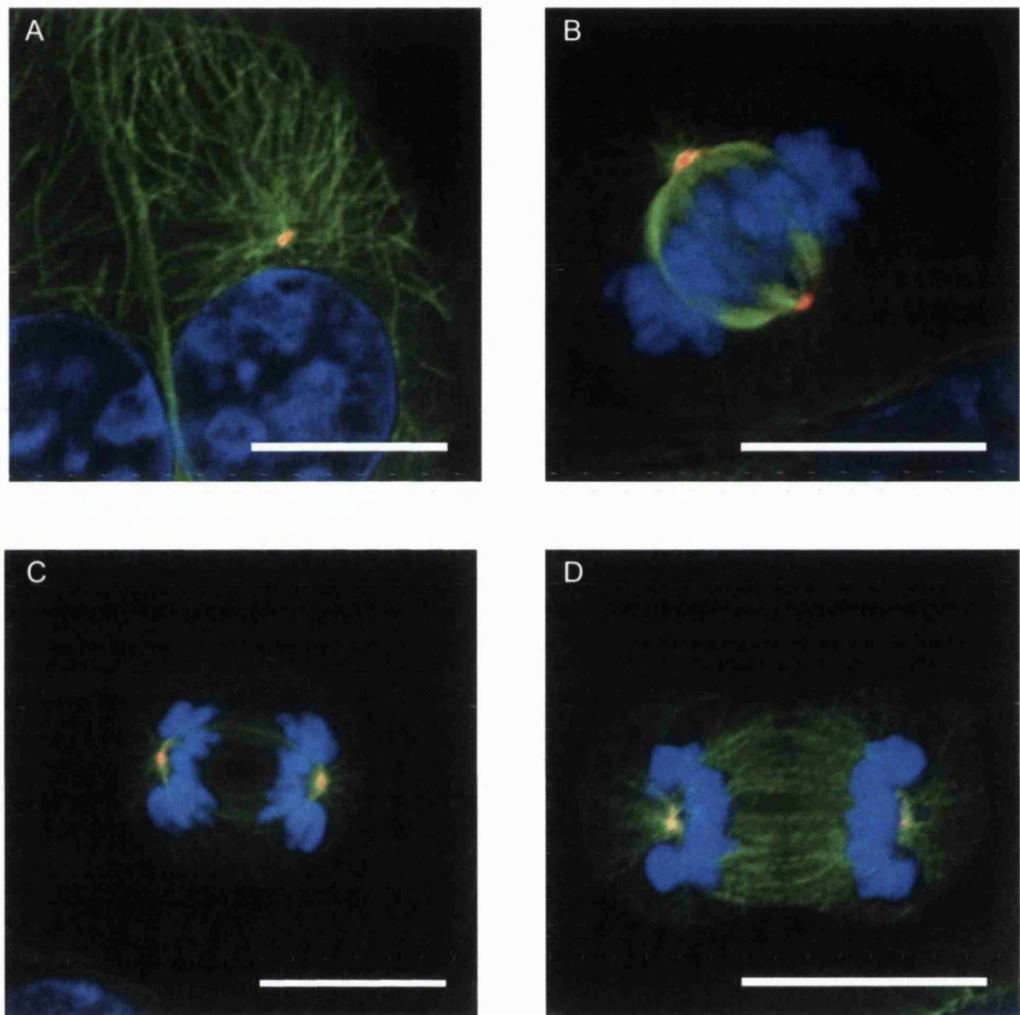


Figure 6.5: The distribution of α - and γ - tubulin in uninfected BHK-21 cells.

BHK-21 cells were plated onto glass coverslips. The cells were mock infected with GMEM and incubated at 37 °C for 24 hours. The cells were fixed using paraformaldehyde and immunolabelled using antibodies against α Tubulin and γ Tubulin. Nuclei/chromosomes were stained using DAPI (blue). In interphase cells (A), the γ Tubulin (red) was found to exist as a single perinuclear dot from which the α Tubulin (green) microtubule network radiated. Image B shows a cell in late metaphase/early anaphase where the chromosomes, attached to the microtubule spindle, have started to migrate towards the spindle poles. Image C shows a cell in late anaphase in which the chromosomes have reached the spindle pole prior to the telophase stage (D) where the nuclear envelope will reform and cytokinesis will occur. Scale bar = 10 μ m

In contrast α -tubulin was conspicuously disrupted in BTV infected cells, although multiple spindles were still present / being formed. However, these spindles appeared less organised and lacked the symmetry of the spindles in uninfected cells (Figure 6.6). The γ -tubulin also appeared ‘smudged’ and divided as opposed to being organised as a punctate dot (Figure 6.6B). However, these data confirmed that each of the multiple spindles contained a MTOC.

It has previously been observed that BTV NS1 clusters towards the nucleus, including around the centrosome (Chapter 4, Figure 4.10 and P. Monaghan, unpublished observations). To investigate if NS1 localised to the disrupted MTOCs in non-mitotic cells, RSAvvvv/16 infected BHK cells were fixed and immuno-labelled using antibodies to NS1 and γ -tubulin (Orab 69 and γ -tubulin respectively, Materials and Methods, Table 2.10). Even in non-mitotic cells the γ -tubulin labelling was disrupted, although it remained in a perinuclear location (Figure 6.7A). NS1 was also found to locate to the same region as the γ -tubulin with each point of γ -tubulin co-localised with NS1 labelling (Figure 6.7B).

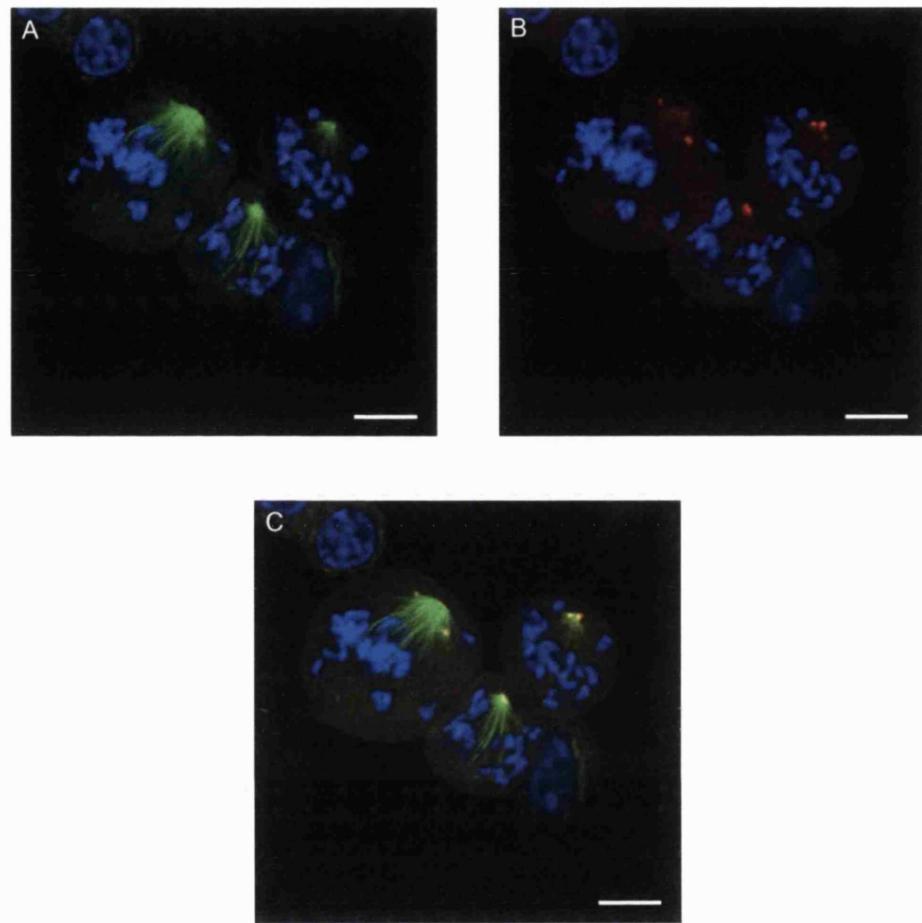


Figure 6: Disruption of α -tubulin and γ -tubulin in cells undergoing aberrant mitosis.

BHK-21 cells were plated onto glass coverslips. The cells were infected with RSAvvvv/16 and incubated at 37 °C for 24 hours. The cells were fixed using ice cold methanol and immunolabelled using antibodies against α Tubulin and γ Tubulin. Nuclei/chromosomes were stained using DAPI (blue).

The α Tubulin labelling (green) revealed a disorganised and non-symmetrical microtubule spindle (A). The γ Tubulin labelling (red) indicated a smudged distribution with numerous intense dots suggesting multiple or divided centrosomes (B). The merged image (C) shows that the γ Tubulin is located towards the pole of the microtubule spindle confirming its position as the MTOC.

Scale bar = 10 μ m

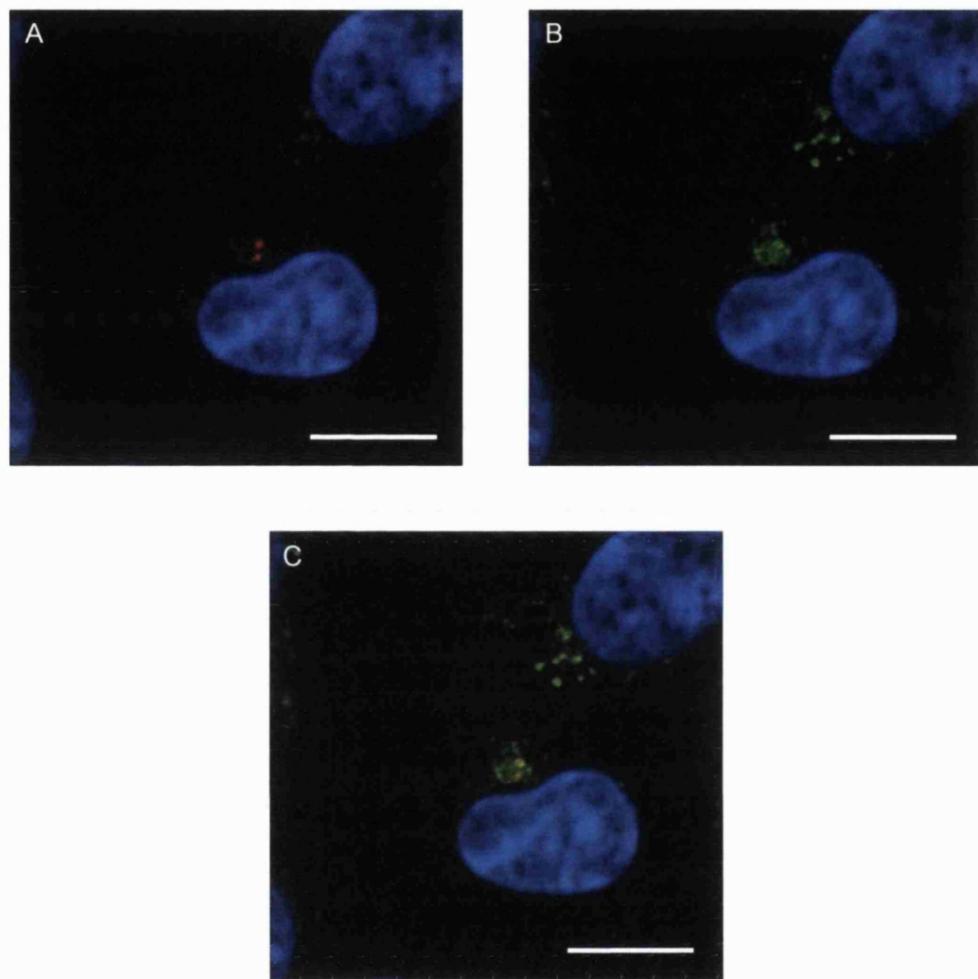


Figure 6.7: Disruption of γ -tubulin in BTV infected BHK-21 cells.

BHK-21 cells were plated onto glass coverslips. The cells were infected with RSAvvvv/16 and incubated at 37 °C for 24 hours. The cells were fixed using ice cold methanol and immunolabelled using antibodies against NS1 and γ Tubulin. Nuclei were stained using DAPI (blue). The γ Tubulin (red) was found to be distributed in a cluster of sever

al dots as opposed to a single perinuclear dot (A). Each dot of γ Tubulin labelling was also associated with NS1 labelling (green, B). The merged image (C) demonstrates that the labelling overlaps.

Scale bar = 10 μ m

6.5: Other proteins involved in mitosis

The apparent failure of correct microtubule – chromosome interactions to form in BTV infected cells, was studied by examination of other proteins that are involved in the mechanism of mitosis.

6.5.1: End binding protein (EB1)

The microtubule associated protein (EB) 1 protein family, is a highly conserved group of proteins which localise to microtubules, with a particular preference for the distal tips of microtubules (Morrison, 2007). EB1 microtubule end labelling can therefore be used to assess the dynamic polymerisation of the microtubules themselves. A loss or re-distribution of EB1 labelling in infected cells might suggest that microtubule dynamism and ability to grow is either lost or altered.

The polymerisation of microtubules was assessed in BHK-21 cells showing aberrant mitosis, at 24 hours post infection with RSAvvvv/16 using confocal microscopy. There appeared to be no significant differences in the labelling of EB1 in infected and uninfected cells (Figure 6.8). In non-mitotic, uninfected cells, EB1 appeared as dashes distributed evenly throughout the cytoplasm of the cells. In uninfected cells undergoing mitosis, EB1 labelling appeared to be more intense and was localised to chromosomes, perhaps reflecting increased activity of the microtubule spindle.

Mitotic cells in RSAvvvv/16 infected cultures, showing an abnormal distribution of condensed chromosomes, also showed intense EB1 labelling, indicating that microtubules were still capable of polymerising (Figure 6.8). However, although the intensity of the chromosomes labelling was largely equivalent to that in uninfected cells, their localisation did not appear to be as focused towards the condensed chromosomes, suggesting that microtubule-chromosome interaction (via the kinetochore) had been lost (Figure 6.8). These data indicate that BTV infection does not inhibit microtubule growth, but does disrupt microtubule-chromosome interactions.

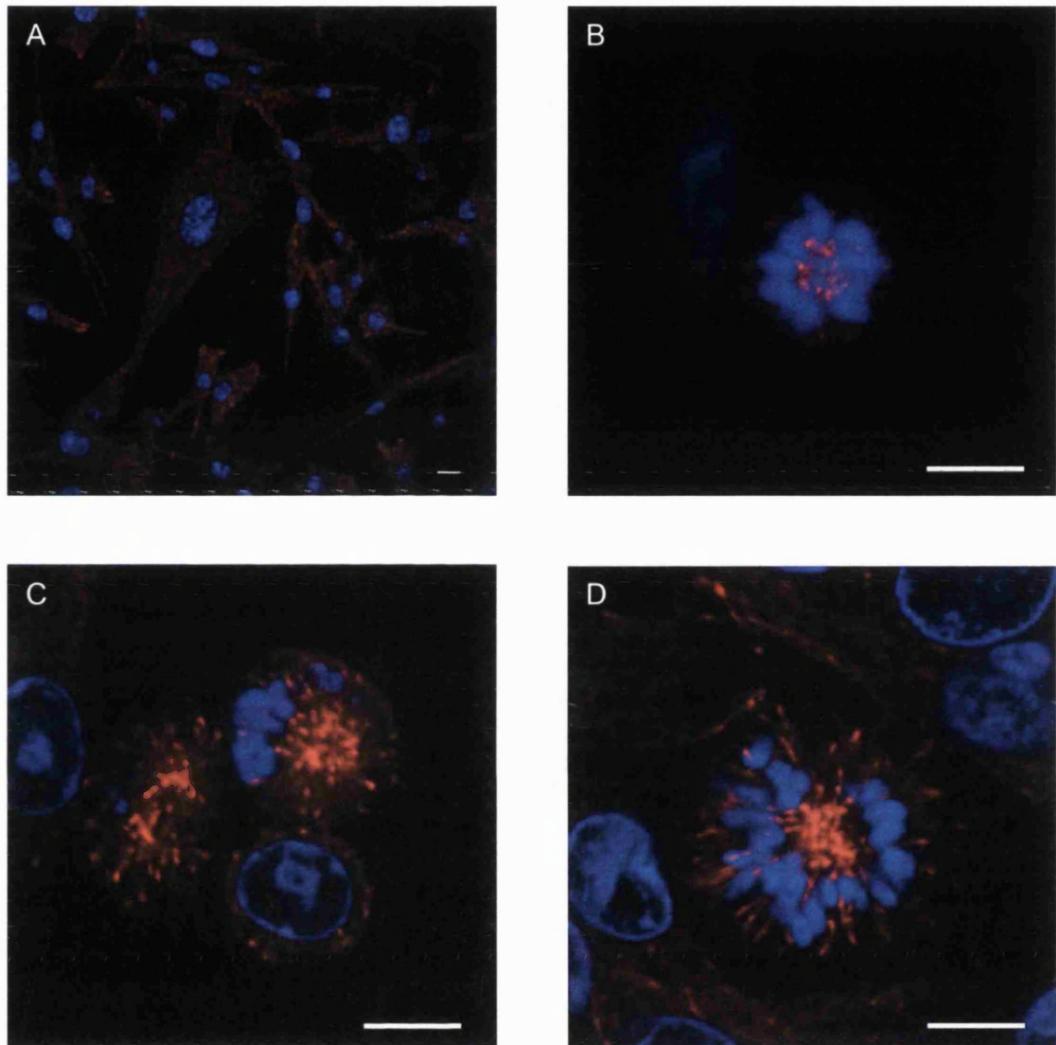


Figure 6.8: Microtubule growth is unaffected in BTV infected mitotic cells.

BHK-21 cells were plated onto glass coverslips 24 hours prior to infection. The cells were mock infected or infected with RSAvvvv/16 and incubated in the presence of 10 % FBS. The cells were fixed at 24 hours post infection and immunolabelled using antibodies to EB1. The nuclei/chromosomes were stained using DAPI (blue). In uninfected non-mitotic cells the EB1 labelling (red) appeared as flecks throughout the cytoplasm (A) but in mitotic cells the labelling was much more intense and concentrated towards the chromosomes (B). In infected cells the EB1 labelling was also intense in the aberrant cell divisions and demonstrated a similar pattern. However, the close association with organised condensed chromosomes was reduced (C and D).

Scale bar = 10 μ m

6.5.2: Centrin

The centrosome is the MTOC of mammalian cells. During the S phase of each cell cycle the centrosome is duplicated to provide MTOCs for the daughter cells. Centrin has a fundamental role in the biogenesis of centrosomes and is therefore a measure of centrosome health. Immunolabelling of γ -tubulin (shown above) indicates that the MTOC is disrupted in BTV infected cells.

Immunolabelling for centrin and NS1 also showed a striking difference between infected and uninfected BHK cells. In uninfected cells centrin was labelled as a punctate dot, adjacent to the nucleus, a typical location for the centrosome (Figure 6.9). However, in infected cells immunolabelling, either indicated that centrin was absent, or it was dispersed in a typical centrosome location adjacent to the nucleus (Figure 6.9). These data agree with those for γ -tubulin, which was also disrupted during BTV infection. However, centrin was also dispersed in infected cells which were not undergoing mitosis, demonstrating that BTV causes disruption of centrosomes in phases of the cell cycle other than mitosis. The most intense accumulations of NS1 appeared to colocalise with the disrupted centrin, although aggregates of NS1 were also observed elsewhere within the cell.

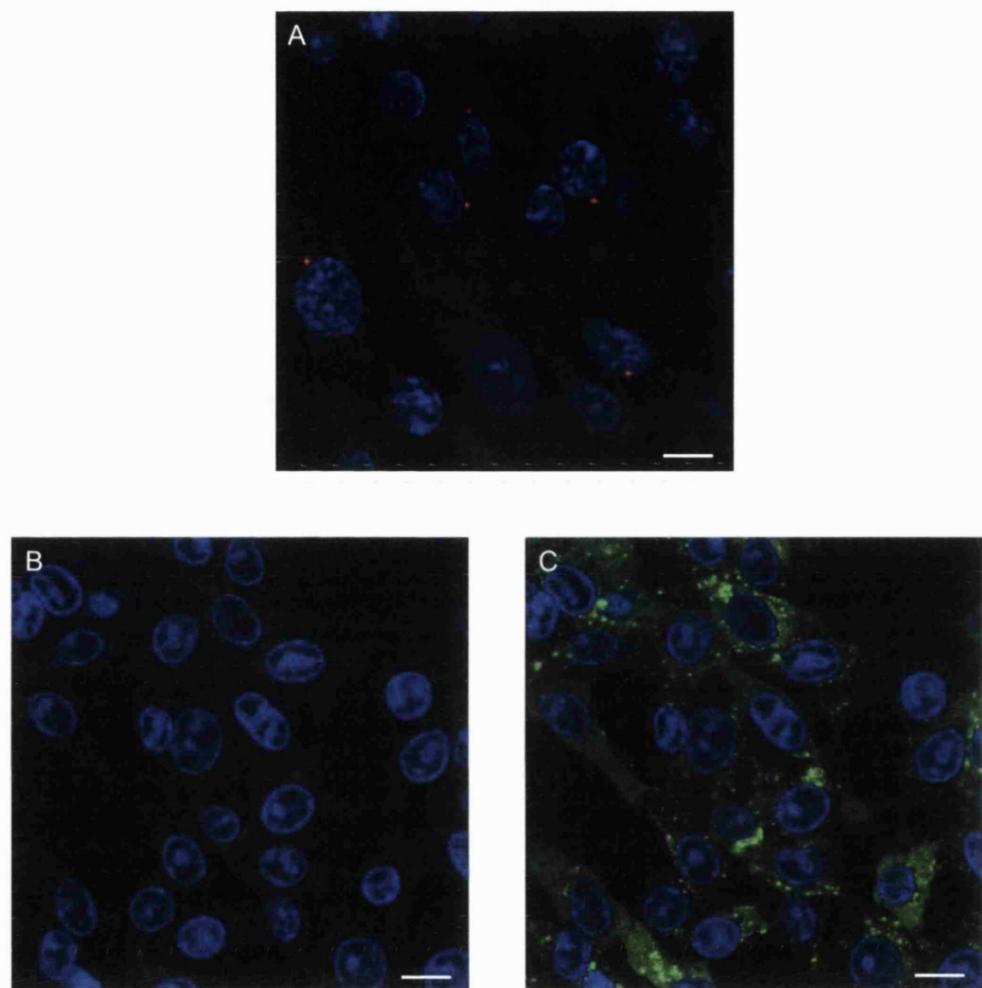


Figure 6.9: BTV infection disrupts centrin.

BHK-21 cells were plated on glass coverslips. The cells were mock infected or infected with RSAvvvv/16 and incubated in the presence of 10% FBS. The cells were fixed using ice-cold methanol at 24 hours post infection and immunolabelled using antibodies to NS1 and α Centrin (labelling the microtubules). In uninfected cells (A), centrin (red) was observed as a punctate dot adjacent to the nucleus (blue) with limited background labelling. In cells infected with BTV, the centrin labelling was weaker and did not occur as a single dot (B). The disrupted centrin labelling appeared to co-localise with the regions of most intense NS1 labelling (green, C).

Scale bar = 10 μ m

6.5.3: FOP

FOP is a centrosome protein that is preferentially located towards the distal end of the mother centriole, and (along with EB1 and p150^{glued}) helps anchor microtubules to the centrosome (Yan et al., 2006). FOP therefore represents another marker for the presence and integrity of the centrosome. In uninfected non-mitotic cells, FOP labelling was observed as a punctate dot adjacent to the nucleus, whereas in mitotic cells two dots were observed. The pattern of the DAPI staining suggested that the two dots in the mitotic cells represented the two spindle poles (Figure 6.10A and 6.10B).

In contrast to the γ -tubulin and centrin labelling in infected cells, the FOP labelling in infected cells remained punctate. However, multiple dots of FOP were observed in the infected cells showing disrupted mitosis (Figure 6.10C and 6.10D). These data suggest that more than one duplication of the centrosome had occurred, indicating interference in the S phase of mitosis. In line with the previous data, NS1 was found to localise to areas of FOP labelling, i.e. the centrosome region, in infected cells.

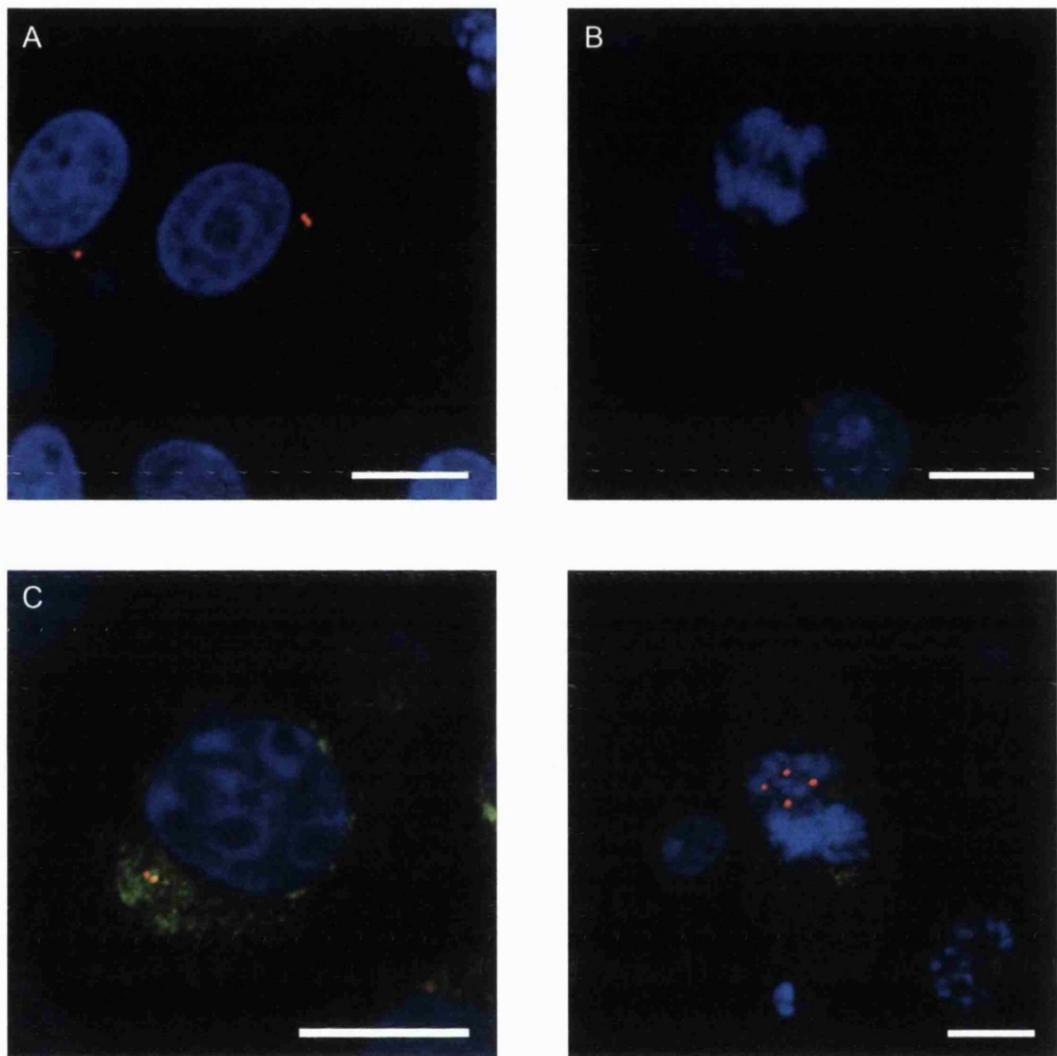


Figure 6. 10: FOP labelling is altered in BTV infected non-mitotic and mitotic cells.
 BHK-21 cells were plated onto glass coverslips 24 hours prior to infection. The cells were mock infected or infected with RSAvvvv/16 and incubated in the presence of 10 % FBS. The cells were fixed at 24 hour post infection and immunolabelled using antibodies to FOP and NS1. The nuclei/chromosomes were stained using DAPI (blue). In uninfected non-mitotic cells, the FOP labelling (red) existed as two extremely close dots adjacent to the nucleus (A). In infected non-mitotic cells the FOP labelling was still intense and punctate but there appeared to be more than the two closely associated dots which were localised in an area of NS1 (C). In uninfected mitotic cells the FOP labelling occurred at two opposite positions suggestive of the MTOC (B) whereas in the equivalent infected cells, several dots of FOP were apparent (D).

Scale bar = 10 μ m

6.5.4: p150^{glued}

In addition to its role at the centrosome, p150^{glued} is an integral component of the dynein complex and is involved in directly binding to the intermediate chains of dynein (Vaughan and Vallee, 1995). Dynein performs critical roles in intracellular transport, including the microtubule spindle organisation (Vaisberg et al., 1993). In uninfected cells immunolabelled for p150^{glued} and NS2, p150^{glued} was found to be distributed throughout the cell in an even but grainy pattern, although intense labelling was also observed in a position assumed to be the MTOC (consistent with the microtubule anchoring role of p150^{glued} at the centrosome, Figure 6.11). During mitosis the p150^{glued} was more concentrated in a pattern resembling the microtubule spindle, in particular towards the spindle poles (Figure 6.11).

In infected (though non-mitotic) cells a dramatic redistribution of p150^{glued} was observed. The p150^{glued} appeared to redistribute into granular masses to one side of the nucleus in a pattern reminiscent of NS1 distribution (Figure 6.12) which, previous data indicate, localise to the region of the centrosome. The NS2 labelling observed in infected cells was occasionally also localised to the area of the redistributed p150^{glued}, although this was inconsistent (Figure 6.12).

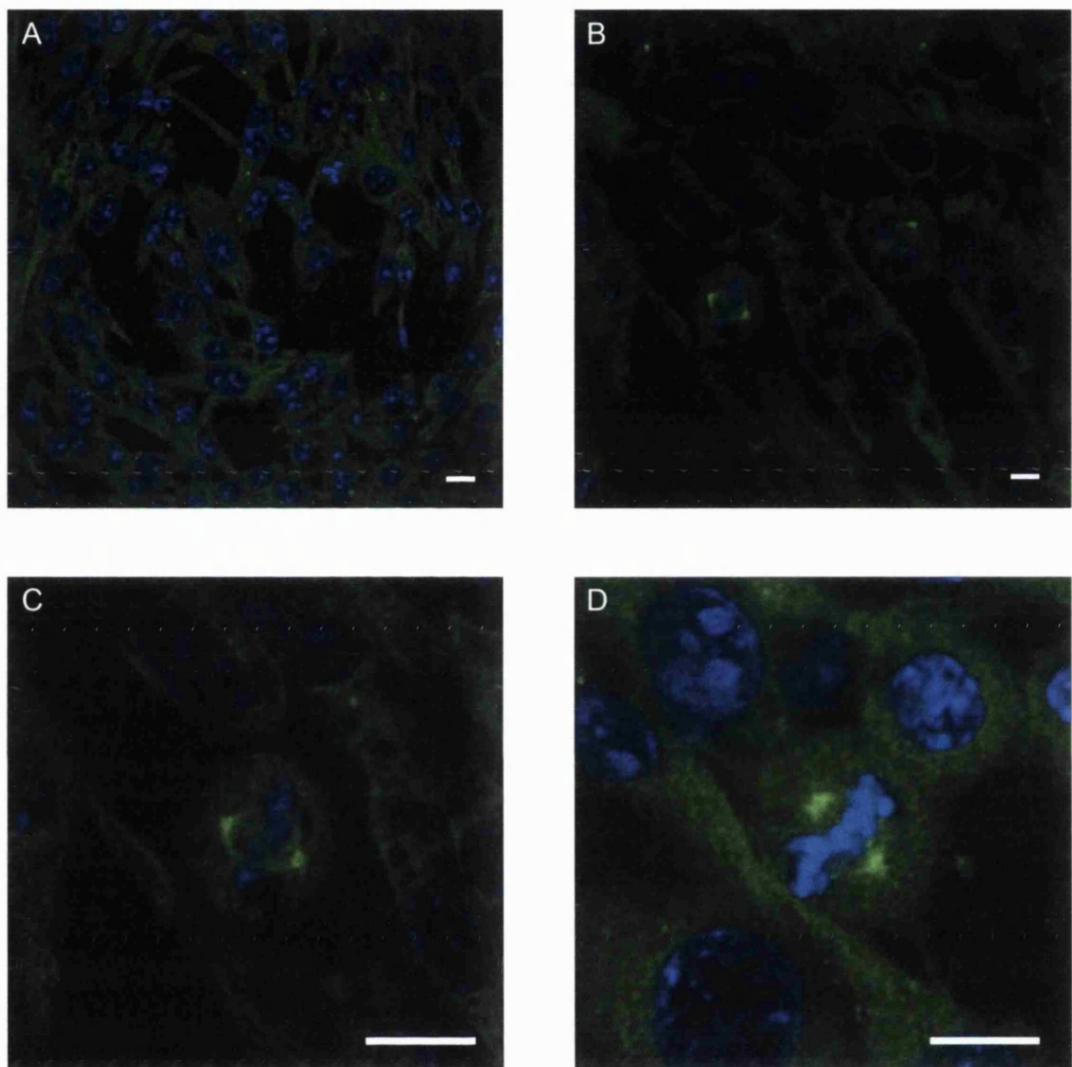


Figure 6.11: The p150^{glued} distribution in uninfected cells.

BHK-21 cells were plated onto glass coverslips. The cells were mock infected with GMEM and incubated at 37 °C for 24 hours. The cells were fixed using ice-cold methanol and immunolabelled using antibodies against p150^{glued}. Nuclei/chromosomes were stained using DAPI (blue). In non-mitotic cells the p150^{glued} labelling (green) was uniformly distributed throughout the cell, occasionally with a more intense perinuclear dot (assumed to be the MTOC, A). In mitotic cells at the metaphase stage the p150^{glued} labelling matched the expected pattern for a microtubule spindle. The labelling was particularly intense towards what was assumed to be the MTOC (based upon the position and the pattern of fluorescence, B-D).

Scale bar = 10 µm

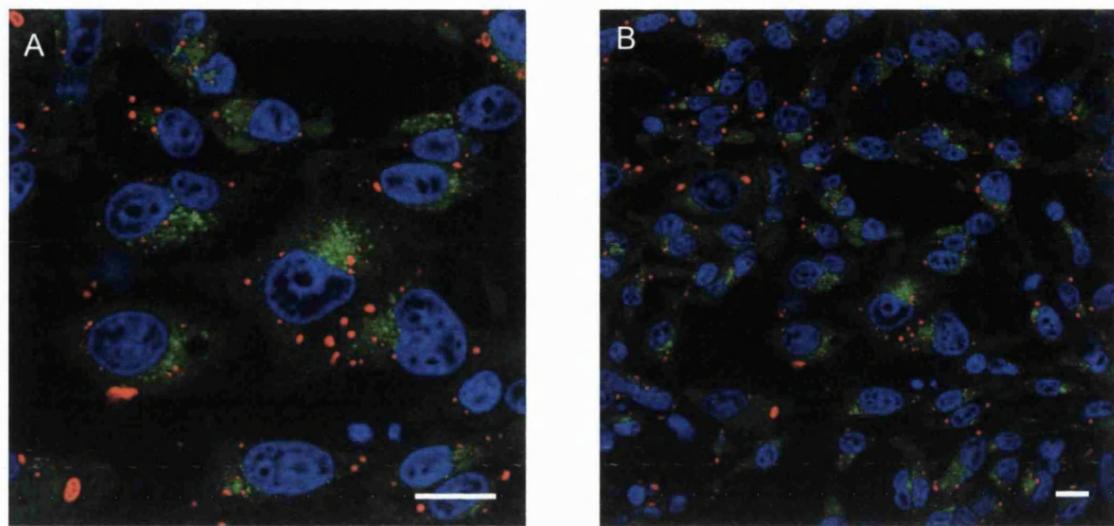


Figure 6. 12: Distribution of NS2 and p150^{glued} in infected cells.

BHK-21 cells were plated onto glass coverslips. The cells were infected with RSAvvvv/16 and incubated at 37 °C for 24 hours. The cells were fixed using ice cold methanol and immunolabelled using antibodies against NS2 and p150^{glued}. Nuclei/chromosomes were stained using DAPI (blue). The NS2 labelling (red) was observed in the form of viral inclusion bodies (A). It was found that the p150^{glued} labelling (green) was relocalised to a perinuclear region (as opposed to the diffuse labelling observed in uninfected cells, Figure 6.11). A low power image (B) revealed that this was a consistent observation in every BTV-infected cell.

Scale bar = 10 µm

6.6: Electron microscopy

One of the consistent observations made by confocal microscopy and immunolabelling of aberrant mitotic cells, was the association of NS2 labelling with the condensed chromosomes (Figure 6.2D). Since the viral inclusion bodies contain a large amount of NS2 particularly around their periphery, these data suggest that VIBs should also be associated with the chromosomes in mitotic cells. Indeed, imaging cells that were observed undergoing abnormal apoptosis by transmission electron microscopy, showed a close association of the VIBs with the chromosomes supporting their hypothesis that they interact (Figure 6.13).

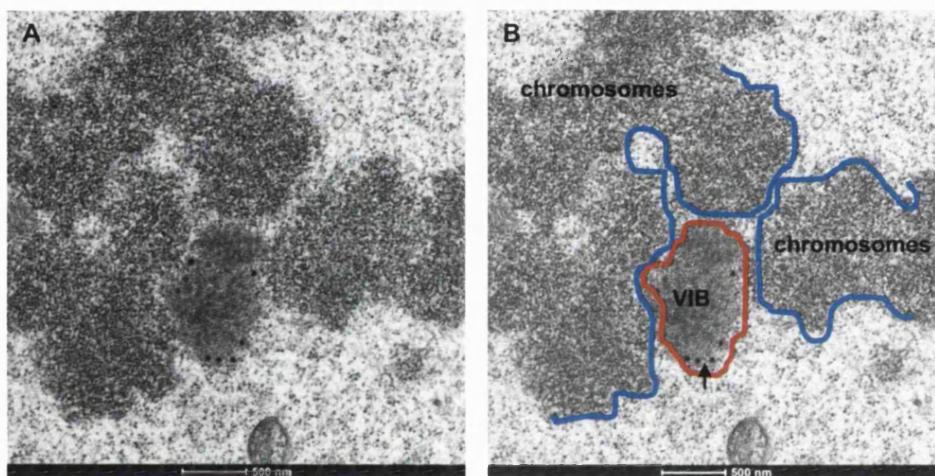


Figure 6. 13: Transmission electron micrograph of a viral inclusion body and condensed chromosomes.

BHK-21 cells were seeded onto Thermanox coverslips (VWR). The cells were infected with RSAvvvv/16 and fixed 24 hours at 37 °C in the presence of 10 % FBS. The cells were fixed using EM fix and prepared for transmission electron microscopy. Micrograph A is a close up image of a BTV infected cell demonstrating the aberrant mitosis. The image shows a viral inclusion body (VIB) nestled between condensed chromosomes distributed in the cytoplasm. Image B is an annotated version of micrograph A highlighting the VIB (outlined in red) and chromosomes (outlined in blue). Virus particles were observed towards the periphery of the VIB as indicated by the arrow.

6.7: Loss of Cytochrome C (an indicator of apoptosis)

The cell cycle is a highly regulated process with multiple ‘checkpoints’ which must be passed for the cell to successfully proceed through cell division (Tyson and Novak, 2008). The precise arrangement of the chromosomes on the mitotic spindle is a critical checkpoint (Ciliberto and Shah, 2009) and one of the outcomes of incorrect spindle formation is apoptosis (Rieder and Maiato, 2004). To investigate whether BTV induced mitotic arrest results in apoptosis, BHK-21 cells infected with RSAvvvv/16 and

cultured in the presence of serum were fixed and immunolabelled with antibodies to NS2 and Cytochrome C (CytC). Cytochrome C is released from the mitochondria when the intrinsic apoptosis pathway is initiated and is therefore a marker for apoptosis (Abu-Qare and Abou-Donia, 2001). In uninfected cells CytC labelling was mitochondrial and distributed throughout the cell (Figure 6.14A). However, in infected cells demonstrating aberrant mitosis, there was a loss of mitochondrial CytC labelling, although this loss was not entirely consistent, and some cells were more affected than others (Figure 6.14B and 6.14C).

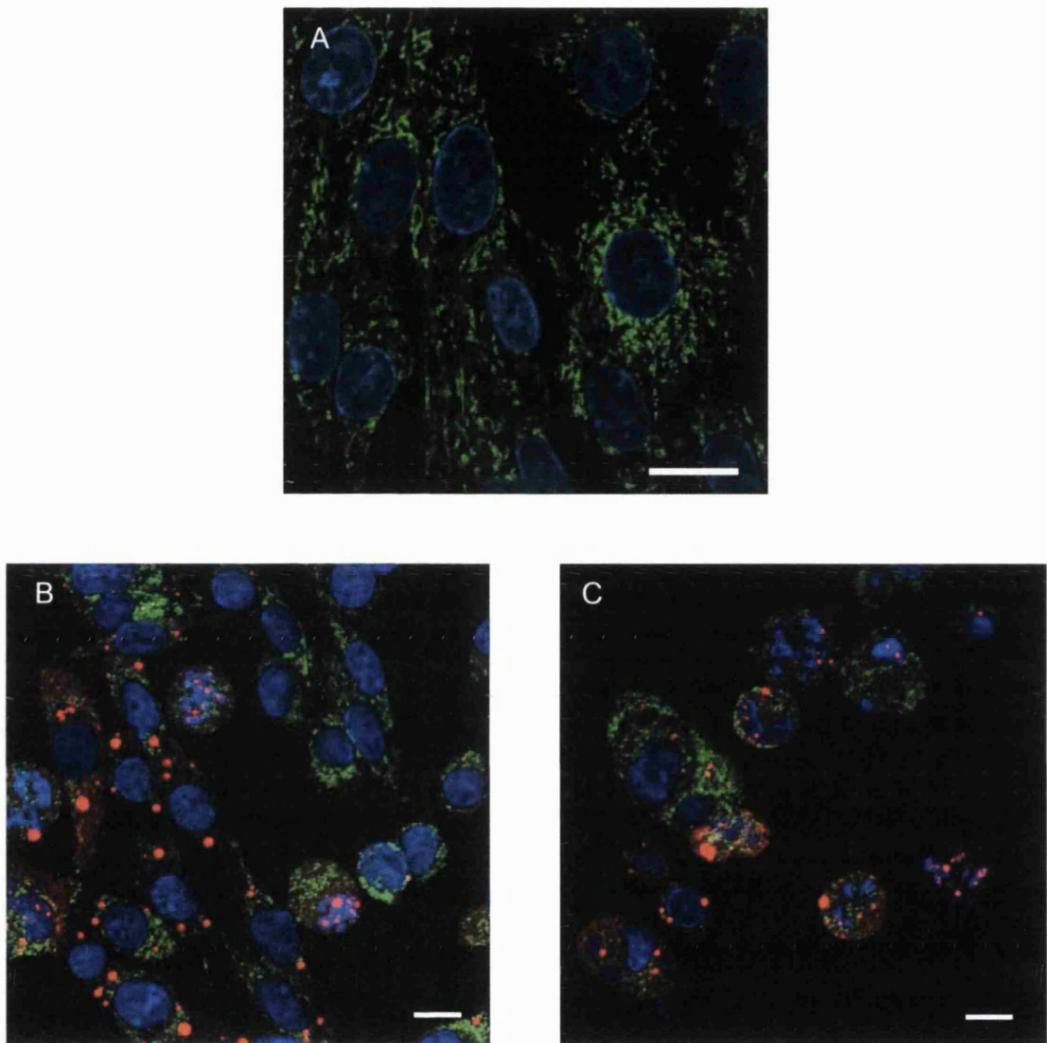


Figure 6.14: BTV infection results in apoptosis.

BHK-21 cells were plated on glass coverslips. The cells were mock infected or infected with RSAvvvv/16 and incubated in the presence of 10 % FBS. The cells were fixed at 24 hours post infection and immunolabelled using antibodies to NS2 and cytochrome C. Nuclei/chromosomes (blue) were stained using DAPI. In uninfected cells (A), the cytochrome C (green) is located in mitochondria resulting in a characteristic mitochondrial pattern. In infected cells (B and C) the cytochrome C labelling is reduced or lost indicating induction of the intrinsic apoptotic pathway. The loss is evident in non-mitotic cells with clear VIBs (red, A) but is more prominent in cells undergoing aberrant mitosis (B).

Scale bar = 10 μ m

6.8: Hypothetical model – NS2 expression and the induction of aberrant mitosis

Confocal and electron microscopy provided evidence that BTV NS2 forms a relationship with the kinetochore of the chromosomes. It is conceivable that the expression of NS2 alone may be sufficient to induce the unusual mitotic events associated with BTV replication in BHK-21 cells. The ORF encoding NS2 was amplified from reverse transcribed RSAvvvv/16 viral RNA and cloned in a manner similar to NS1 and NS3 (Chapter 4, sections 4.3.1 and 4.5) with C-terminal V5 and 6HIS epitopes. The resulting plasmid (pcDNA3.1 V5/HIS_NS2) was transfected into uninfected BHK-21 cells, which were incubated for 24 hours at 37 °C for expression to occur. The cells were fixed using paraformaldehyde and immunolabelled with antibodies targeting V5 and α -tubulin. NS2 was detected as small aggregated structures associated with microtubules, in a manner reminiscent of the early stage of VIBs formation in BTV infected cells (Ross-Smith, 2008) (Figure 6.15A and 6.15B). The number of cells that were successfully transfected with the plasmid and in the process of mitosis was low. It was therefore difficult to determine the effect that NS2 expression has on dividing cells. However, cells showing aberrant mitosis, were detected and each one contained evidence of NS2 expression. The presence of multiple spindles was seen in these cells, as alignment of the chromosomes in parallel to (rather than across) the microtubule spindle (Figure 6.15C). Interestingly, NS2 labelling appeared evenly distributed throughout these dividing cells, with little if any evidence of VIB-like structures (Figure 6.15).

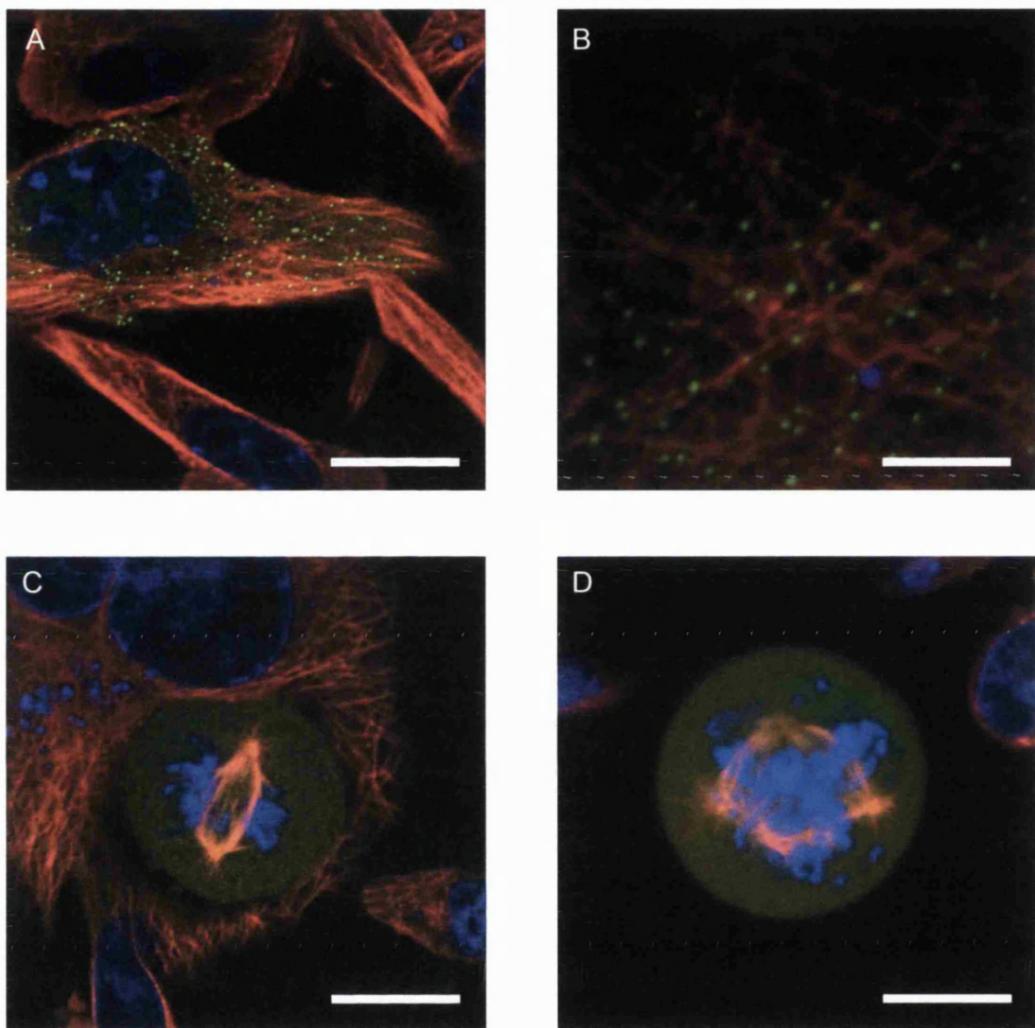


Figure 6.15: NS2-V5 localises to microtubules and induces aberrant mitotic events. BHK-21 cells were plated onto glass coverslips. Plasmid pcDNA3.1V5/HIS_NS2, expressing the RSAvvvv/16 NS2 protein fused to a V5 epitope, was transfected into uninfected BHK-21 cells and incubated at 37 °C for 24 hours. The cells were fixed and immunolabelled using antibodies against the V5 epitope and α Tubulin. Nuclei/chromosomes were stained using DAPI (blue). NS2-V5 (green) was observed as small dots distributed throughout the cell (A, scale bar = 10 μ m) which, under high power magnification (B, scale bar = 1 μ m) were localised to the microtubules (red). In transfected mitotic cells the NS2 labelling appeared uniformly diffuse throughout the cell (C and D, scale bar = 10 μ m). The mitotic cells had features reminiscent of BTV induced mitotic arrest, notably the parallel distribution of chromosomes relative to the microtubule spindle (C) and multiple spindles (D).

Scale bar = 10 μ m

6.9: Discussion

The inclusion of serum in the cell-culture media during BTV infection, led to accumulation of cells that were arrested in mitosis. The most likely reason for this is that in the absence of serum, cultured cells rarely divide. Further investigation showed that in these cells the mechanism of mitosis was abnormal with multiple microtubule spindles and disorganised distribution of the condensed chromosomes.

BTV-NS2 was associated with condensed chromosomes in these mitotic cells, while NS1 was localised to the centrosome in non-mitotic cells. A high level of variation in the number of mitotic cells per field of view was detected in infected cultures. One explanation may be uneven infection across the coverslip, and in previous experiments using a low multiplicity of infection BTV established islands of infection, which subsequently expanded (data not shown).

Although infection of BHK-21 cells by BTV strain RSAvvvv/16, caused more cells to enter mitotic arrest, this effect was also observed with the BTV-1 reference strain (RSArrrr/01) and the UK BTV-8 field strain (UKG2007/34). BHK-21 cells are particularly susceptible to BTV and may be inherently more sensitive to interference in the mechanism of mitosis (McPhee et al., 1982), although this effect was also observed in Vero and BPAEC cells. Since both field strains and cell culture adapted strains interfere with mitosis in several mammalian cell types, this appears to be real and a common property of BTV.

Cytochrome C labelling showed that BHK-21 cells undergoing aberrant mitosis, were also in the process of apoptosis, and the incidence of apoptosis increased with the frequency of cell cycle arrest. This study indicates that apoptosis in BTV infected cells, can be induced as a response to the aberrant mitosis induced by BTV infection (particularly BTV-NS2), as opposed to being a response to the outer capsid proteins, as previously reported by (Mortola et al., 2004).

During the course of these studies, BTV was shown to affect two aspects of the mitosis mechanism in infected cells. Firstly, it disrupts the formation of microtubule spindles, and secondly it blocks or disrupts the interaction between the microtubules and the condensed chromosomes.

The centrosome was disrupted in BTV infected cells that were not undergoing mitosis, which would inevitably also affect spindle formation. It cannot be determined from these data whether the disruption of the centrosome by BTV instigates a cell to undergo mitosis. The fact that the aberrant mitotic cells were observed suggests that the

disruption does not prevent the cell from entering mitosis. The localisation of NS1 to components of the centrosome and the surrounding region within the cell is intriguing. It is tempting to speculate that NS1 is at least in part responsible for disruption of the centrosome. It is conceivable that the components of the centriole are simply split apart, resulting in the appearance of several MTOCs. A possible outcome of centriole disruption is the presence of supernumerary procenitrioles and subsequently supernumerary MTOCs and several spindles. Further studies, including the expression of NS1 in isolation, are required to further study this possibility.

It appears likely that disruption of spindle assembly may be at least partly caused upon disruption of the centrosome, as a result of interactions with NS1. However, NS2 also physically blocks the kinetochores and may be a second, more direct cause for aberrant cell division. If microtubules cannot bind to the kinetochore because NS2 interacts with a kinetochore protein, then the chromosomes will be 'pushed' aside, potentially resulting in the arrangement of the chromosomes parallel to the spindle, as seen in the BTV infected and mitotic cells.

This outcome would clearly fail the spindle 'checkpoint' during mitosis, which requires every kinetochore to be attached in a bipolar manner, with equal force towards each spindle pole (Rieder et al., 1995; Rieder and Maiato, 2004). These data and observations of apoptosis, suggest that aberrant mitosis in BTV infected cells may represent an example of 'mitotic catastrophe' (Castedo et al., 2004).

The nature of the protein which, based upon the hypothesis proposed here, binds to and blocks the kinetochore is not clear, although a possible candidate is the dynein/dynactin complex. Dynein forms a key interaction with the kinetochore (Varma et al., 2008) and several viruses including poliovirus, hantaan virus, herpes simplex virus type 1 and hepatitis E virus, have all been shown to utilise dynein to interact with the cellular transport machinery (Dohner et al., 2005; Dohner et al., 2006; Gonzalez Duran et al., 2007; Kannan et al., 2009; Ramanathan et al., 2007). The human papillomavirus protein E7, has been implicated in induction of aberrant mitotic events, by interacting with the nuclear mitotic apparatus protein 1 resulting in delocalisation of dynein (Nguyen and Munger, 2009). The *Tobacco mosaic virus* 'movement-protein' has also been shown to reorganise microtubules and disrupt the centrosome, although its impact upon mitosis was not investigated (Ferralli et al., 2006).

Alternative non-dynein proteins that might bind to and block the kinetochore include the kinesins and other microtubule end binding proteins (Morrison, 2007;

Santaguida and Musacchio, 2009). Kinesins are classically plus-end directed proteins i.e. target the growing tip of the microtubule, generally towards the cell periphery. Several viruses have been shown to require a kinesin for their lifecycle. Vaccinia virus and African swine fever virus have both been shown to utilise kinesin-1 for egress of viral particles from the cell (Jouvenet et al., 2004; Rieddorf et al., 2001). BTV NS2 in the form of VIBs tends not to migrate towards the cell periphery, although it does accumulate as VIB within the cell, and individual VIBs may also come together and fuse at later stages of replication (Brookes et al., 1993; Ross-Smith, 2008).

Electron and confocal microscopy, and data from expression of NS2 (from a transfected plasmid) in uninfected cells, provides evidence that NS2 can induce cell cycle arrest. In non-mitotic infected cells NS2 appears to be associated with microtubules, consistent with NS2 interacting with a microtubule associated protein. However, the distribution of NS2 labelling in mitotic cells was diffuse as opposed to VIB-like. This may relate to the phosphorylation status of NS2 protein as only phosphorylated NS2 has been shown to aggregate to form VIB-like structures (Modrof et al., 2005). VIBs were observed in infected cells undergoing mitosis reflecting the complex nature of authentic VIBs containing many other components in addition to NS2.

The impact of BTV infection on mitosis in insect cells was not investigated during this project. The results of such an investigation would be of considerable interest, as adult *Culicoides* suffer few effects as a result of infection.

The induction of cell arrest in mitosis by BTV is clearly different to the G2/S phase block induced by Reovirus (Poggioli et al., 2000). However, the data described here for BTV relate well to a study by Hu *et al* (2008) using cancer cell lines. The authors found that BTV-10 was cytotoxic in cancer cells and induced a sub-G1 cell cycle arrest, although it is not stated whether infected cultures were cultured in the presence of serum (Hu et al., 2008).

The relevance and requirement for mitotic arrest during BTV replication cycle is debatable. BTV replicates efficiently in the absence of serum and therefore in the absence of cell division. In addition, the endothelial cells which BTV infects in the ruminant host divide slowly. The arrest of mitosis may therefore represent a consequence of utilising a microtubule interacting protein, e.g. dynein, which is critical for cell division, and may enhance the replication efficiency of BTV in lytically infected mammalian cells.

Future work on this project will involve identifying and characterising the binding partner for BTV-NS2. Collaboration has been established with Leeds Institute of

Molecular Medicine where attempts will be made to co-immunoprecipitate NS2 interacting proteins using the plasmid pcDNA3.1V5/HIS_NS2 assembled as part of this project.

Chapter 7: Final Discussion

7.1: Introduction

A major objective of this project was to investigate the role of BTV non-structural proteins during infection and replication in mammalian cells. The project was designed to test the published hypothesis that the relative levels of NS1 and NS3/3A determine the outcome of infection in mammalian cells (Owens et al., 2004). There have been several reports of NS3/3A involvement in virus release from infected cells and in determination of pathogenicity/virulence (Celma and Roy, 2009; Hyatt et al., 1993; Meiring et al., 2009; O'Hara et al., 1998; Wirblich et al., 2006).

The role of NS1 is less well defined, although inhibition of tubule assembly is reported to have resulted in a non-cytolytic infection (Owens et al., 2004). The precise role(s) of the remaining non-structural protein, NS2, has been widely discussed (Ross-Smith, 2008; Taraporewala et al., 2001; Thomas et al., 1990) but remain uncertain. However its ability to form viral inclusion bodies (VIBs) and its ssRNA binding capabilities suggest that NS2 primarily functions in virus replication and assembly.

7.2: A real-time RT-PCR assay for BTV

A significant outcome of the project was the development of an effective real-time RT-PCR assay for detection of BTV RNA in experimental or clinical samples. The selection of Seg-1 as an assay target was novel, as it had not previously been used by any of the published RT-PCR assays. Seg-1 was chosen because of its very highly conserved nature (as the viral polymerase gene) making it suitable as a target for real-time PCR TaqMan probes, which are notoriously sensitive to mismatches (Jimenez-Clavero et al., 2006; Yao et al., 2006).

The resulting assay (Chapter 3; Shaw et al 2007) proved to be both specific and sensitive, detecting all of the BTV strains that were analysed from the IAH reference collection (including reference strains of BTV-1 to 24), but not other closely related orbiviruses (such as EHDV or AHSV). Most recently a novel strain of BTV was detected in Switzerland (Toggenburg Orbivirus [TOV]) which was not detected by the assay. This virus has been tentatively identified as BTV-25, and the primers / probes used in the assays have been re-designed, to include extra redundancies, which should allow

detection of TOV Seg-1. These studies are ongoing by members of the Arbovirus Molecular Research Group at IAH and results are not yet available

At the beginning of the project, no rRT-PCRs for detection of the BTV species had been published. Very few of the conventional gel-based RT-PCR assays that were available, were capable of detecting all BTV isolates (Anthony et al., 2007). Methods for identification of pathogens are continually being developed and modified. However, rRT-PCR remains a gold standard for molecular diagnostics, and the development of the Seg-1 specific rRT-PCR assay represents a significant step forward in the diagnosis of BTV.

Although its use in the project was ultimately rather limited, the rRT-PCR assay has been used extensively during the northern European BT outbreak and now represents a front line test for BTV detection by the Community Reference Laboratory for BTV at IAH Pirbright, and in several other diagnostic laboratories across Europe. The introduction of RT-PCR technology for diagnosis has increased the reliability and speed of BTV detection and characterisation. The assay has also been used in other projects, including an animal experiment by Darpel *et al* (2007) using the northern European BTV-8 strain.

Other ‘type-specific’ RT-PCR assays have subsequently been designed to determine BTV serotype (Maan et al., 2007a; Mertens et al., 2007). These ‘conventional’ gel based assays can be coupled with nucleotide sequencing of the amplified products to give even precise characterisation of novel BTV isolates. Sequence analyses and phylogenetic comparisons can be used to identify individual virus strains and lineages in a manner that was previously impossible by serological methods alone. However, sequencing remains more cumbersome and protracted, and real-time RT-PCR is likely to be the method of choice for the foreseeable future.

Commercialisation of the BTV-group-specific real-time RT-PCR assay targeting Seg-1 (<http://www1.qiagen.com/Products/cadorBTVRT-PCRKit.aspx>) that was developed during this project represents an important advance in the quality control and inter-laboratory concordance achievable by BTV RT-PCR. In particular it has allowed harmonisation of assay sensitivity for BTV detection between the different laboratories, which use a published version of the assay. The assay appears to be robust and can detect a diverse array of different BTV strains. However, it is important that the primers and probes are checked against new isolates as they become available. This allows any potential problems with detection to be identified and, if necessary, the primers and or probe used in the assay to be modified, (e.g. as with TOV).

7.3: Silencing of BTV mRNAs

Artificially produced dsRNA copies of BTV genome segments 5 and 10 were treated with Dicer, generating siRNAs, which were lipofected into BTV infected cells. Although siRNAs generated from *in vitro* transcribed dsRNA representing Seg-5 were capable of silencing expression from a co-transfected plasmid expressing NS1, no change in the CPE caused by BTV was detected. The experiment would be expected to knock down the level of NS1 therefore increasing the level of NS3/3A relative to NS1. Taken in isolation, this result appears to contradict the hypothesis from Owens *et al* (2004) that the presence of high levels of NS1 is responsible for CPE in BTV infected mammalian cells.

Time restrictions have prevented any further experimentation using siRNAs. However, a collaboration with the Centre for Drug Delivery Research at the School of Pharmacy, University of London, is utilising siRNAs generated during this project to assess next generation delivery mechanisms for use in RNAi therapeutics.

Many viruses have mechanisms to circumvent RNA silencing (RNAi) (Li and Ding, 2006) and BTV may also have an anti-RNA silencing response. Anti-RNAi strategies are often based on binding dsRNA and therefore hiding it from the dsRNA surveillance systems within the host cell (Li and Ding, 2006). There are reports that orthoreovirus capsid protein sigma 3 (σ 3) represses the PKR response by binding dsRNA, and may play an anti-RNAi role in the same way (Lichner *et al.*, 2003; Yue and Shatkin, 1997). The surface protein of BTV cores (VP7) also binds dsRNA (Diprose *et al.*, 2002). The binding of dsRNA by capsid proteins may therefore represent a general mechanism by which reoviruses avoid host anti-dsRNA responses. However, it has been suggested that the viral helicase VP6 could also function as an anti-RNAi protein (P. Mertens, H. Attoui, personal communication), which could eliminate siRNAs by maintaining them in a single stranded form that would be more susceptible to degradation.

Partly because of problems with the availability of laboratory space to work with infectious BTV at IAH Pirbright, during the period of this project, there was insufficient time to carry out all of the planned experiments to alter NS3/3A and NS1 expression levels. In view of the potential for a BTV anti-silencing mechanism, further work, will be required to confirm that the silencing strategy used is effective at reducing the level of NS1 in BTV infected mammalian cells. The importance of the higher relative levels of NS3/3A expressed in BTV infected insect cells has not been determined and equivalent

experiments will need to be carried out to knock down NS1 and/or NS3/3A in this system. The presence of a BTV anti-RNAi response could also be tested using commercially available control siRNAs, against specific host cell mRNAs, e.g. glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative levels of NS1 and NS3/3A should also be analysed in $\gamma\delta$ T cells, which can become persistently infected with BTV (Takamatsu et al., 2003).

7.4: The intracellular expression of BTV protein

The transfection of plasmids into mammalian cells was designed to allow the balance of NS1 or NS3/3A to be altered. These plasmids were successfully assembled and expression analysis confirmed that NS1 and NS3/3A were expressed effectively after their transfection into uninfected cells. The localisation of the expressed proteins matched their previously reported distribution (Bansal et al., 1998; Eaton et al., 1988; Hyatt et al., 1991). Epitope-tagged NS3/3A was clearly associated with membranous components of the cell, in particular the Golgi apparatus and the plasma membrane (Bansal et al., 1998; Hyatt et al., 1991). NS3/3A-EGFP also appeared as clusters on the plasma membrane, presumably at the sites of virus release (Hyatt et al., 1991; Hyatt et al., 1993). Epitope-tagged NS1 localised towards a perinuclear position and, more specifically, to components of the cytoskeleton, e.g. the MTOC (Eaton et al., 1988, P. Monaghan, unpublished observations).

Owens et al (2004) suggested that both NS3/3A and NS1 contribute to CPE and the outcome of infection and therefore may be expected to interact within mammalian cells. It is significant that in this study, the two proteins do not co-localise, but instead are directed to distinct locations within the cell. These data argue against direct interaction, and instead point to each protein performing separate functions which, together result in the relative presence or absence of CPE.

The transfection of plasmids expressing tagged versions of NS3/3A in uninfected cells itself appeared to cause CPE, as compared to cells transfected with only the plasmid backbone. These data were not recordable but indicate that, contrary to the suggestion that NS3/3A promotes a non-lytic infection, the protein has a more destructive role in mammalian cells. The apparent disruption caused by NS3/3A, could be explained by its insertion into cell membranes associated with the secretory pathway and its role as a viroporin (Bansal et al., 1998; Han and Harty, 2004). The CPE observed after expression

of NS3/3A in mammalian cells also fits with the demonstration that NS3/3A of AHSV is cytotoxic (Meiring et al., 2009; van Niekerk et al., 2001).

A lack of NS3/3A dynamism was observed by live cell imaging of transfected /infected cells. There is a large body of published literature indicating that the role of NS3/3A is in virus release (Beaton et al., 2002; Celma and Roy, 2009; Guirakhoo et al., 1995; Hyatt et al., 1993; Wirblich et al., 2006). Whilst not contradicting these reports, the results obtained here suggest that NS3/3A-mediated release may be a slow event.

The expression plasmids were also transfected into BTV infected mammalian cells. However, very few infected cells also expressed the fusion proteins. Protein expression from the transfected plasmids, was subject to shut-off as a result of BTV infection in mammalian cells, in a similar manner to the host cell proteins. This result suggests that expression of specific proteins from these plasmids would be difficult or impossible in the presence of BTV infection. However, these data confirm that BTV interrupts the host cell protein expression pathway and prompted an investigation into the mechanism of BTV mRNA translastion.

7.5: The translation of host vs. BTV mRNAs

Investigation into the translation of BTV-like and mammalian-like mRNAs revealed that the untranslated regions are important for translation of BTV mRNAs. Both UTRs are required for translation, as the removal of either prevented translation. Several aspects in the 3'UTR were found to be of particular importance, particularly the terminal hexanucleotide, a need for the 3' terminus to be exposed, and a requirement for other parts of the UTR. Poly(A) dependent translation was also shown to be severely inhibited in BTV infected cells. These data indicate evolutionary pressure for BTV to maintain several features of their genome segments / mRNAs, to maintain viability. They also reveal certain similarities between the replication strategy used by different genera of the family *Reoviridae*.

The BTV protein expression plasmids that were transfected into mammalian cells generated mRNAs which contain the 5' UTR of the relevant BTV genome segment. The only differences between the plasmid-derived transcripts and authentic viral transcripts are therefore the location of transcription (nucleus vs. cytoplasm) and the presence of a 3' poly(A) tail in place of the viral 3' UTR. Previous data for BTV and other viruses has suggested that inhibition of host cell expression occurs at the level of translation. The critical importance of the poly(A) tail in translation of host cell mRNAs and its absence

in viral transcripts makes it an attractive target for viral intervention in host gene expression

There was a clear correlation between the amount of transfected NS2 mRNA, and the efficiency of translation from a co-transfected reporter mRNA. This suggests that NS2 may act in a similar manner to rotavirus NSP3. In the NSP3 model, an asymmetric homodimer of NSP3 binds specifically to RV RNAs. The dimer forms a tunnel within which the terminal nucleotides (3'-UGACC-3') bind, whilst concurrently binding eIF4G, circularising the RNA in a manner similar to the way PABP enhances translation of polyadenylated mRNAs (Groft and Burley, 2002). The greater affinity of NSP3 compared to PABP for eIF4G leads to the exclusion of PABP from the translation initiation complex, resulting in a simultaneous shut-off of poly(A) dependent translation (Piron et al., 1998).

The NS2 protein of BTV possesses several characteristics that appear distinct from RV NSP3, e.g. the possession of nucleotide triphosphotase activity and viral inclusion body formation. However, like NSP3, NS2 can multimerise and also has ssRNA binding capabilities. Previous work has also suggested that NS2 performs a role in mRNA transport (Ross-Smith, 2008). The data obtained in Chapter 5 clearly demonstrate a link between the amount of NS2 and the efficiency of BTV-like reporter mRNA translation. These data support the hypothesis that NS2 plays a major role in translation.

There is a striking similarity between BTV and rotaviral mRNAs. Firstly, both types of mRNA possess eukaryotic-like methyl cap structures, are non-polyadenylated, and have a linear arrangement of 5'UTR-open reading frame-3'UTR. Of particular significance for the NSP3 model of viral protein translation/host translation inhibition, is the precision of the 3' terminal regions. In this respect, the BTV mRNAs closely resemble rotaviral mRNAs by having 3' terminal bases that are common to every segment across the entire virus species. Both BTV and RV mRNAs terminate in an exposed cytosine. The terminal cytosine of RV mRNAs was shown to bind deep inside the NSP3 homodimer (Deo et al., 2002). The similarity of the mRNAs, together with the observation that NS2 performs a role in translation, makes it tempting to speculate that a functional homologue to PABP may be a wider feature of reovirus translation mechanisms.

7.6: The differences between BTV and rotavirus

The role of NSP3 in rotavirus translation has recently been questioned (Montero et al., 2006). It was found that, although NSP3 was required for the shut-off of poly(A) dependent translastion, viral proteins were still synthesised when the level of NSP3 had been reduced by RNA silencing (Montero et al., 2006). Furthermore, it is noteworthy that this model requires capped mRNA, whereas it is thought that reovirus and BTV switch from cap dependent to cap independent, or 'non-cap' dependent translation during infection (Skup and Millward, 1980; Stirling, 1996). It is apparent that the translation mechanism of these viruses requires more research and, in particular, the form of translation that occurs at particular points during the infection. A siRNA study to determine the proteins required for the translation of BTV proteins e.g. eIF4G would be of particular interest.

The data obtained in this project does not wholly support the hypothesis that NS2 acts in a similar fashion to NSP3. Whilst the enhancement of translation was clear, BTV NS2 did not inhibit poly(A) dependent translation, in the same manner as NSP3.

The ability of BTV to inhibit translation in mammalian cells, while leaving insect cell translation unaffected, is an intriguing problem. In order for the virus to avoid suppressing synthesis of its own proteins, there must be a level of control regarding which expression systems are repressed. A level of control could potentially exist due to differences in the affinity of BTV proteins, e.g. NS2, for the insect and mammalian homologues of a particular host molecule, e.g. PABP. A more detailed understanding of the translation process could clarify how this control is achieved. However, it is also possible that expression control (wholly or partially) occurs at a point other than translation (for example at the level of transcription).

Initial work in this project investigating transcription from plasmids during BTV infection indicated that mRNA synthesis is inhibited in BTV infected cells (data not shown). These data concur with previous work which demonstrated shut-off of RNA synthesis (Huismans, 1970b). In contrast to the mammalian RNA polymerase II, the BTV polymerase is resistant to actinomycin D (P. Mertens, personal communication). The properties of these enzymes are clearly different, suggesting that host cell expression could potentially also be controlled at the level of transcription. Interestingly, such a system has been shown for another arbovirus, Bunyamwera virus, which displays similar cytopathology characteristics to BTV in mammalian and insect cells (Thomas et al.,

2004). It is possible that controls exist at both levels, as the speed of shut-off achieved from the inhibition of transcription alone is likely to be slower than that observed as a result of BTV infection in BHK-21 cells.

7.7: The impact of BTV on the host cell cycle

There are many descriptions of viruses affecting the cell cycle in a variety of ways. It could be beneficial for a virus to block the cell cycle in a stage which particularly favours virus replication. In particular, stages such as S phase and mitosis there may be an abundance of energy and resources available that could enhance BTV replication. However, BTV can replicate effectively in the absence of cell division, for example when cells are cultured in the absence of serum, demonstrating that mitosis is not a prerequisite for replication.

Aberrant cell division was observed in BTV infected cells, or cells expressing NS2, which may reflect the use of particular microtubule end motor protein(s), e.g. dynein/dynactin, for intracellular transport of viral components. The cytoplasmic nature of such proteins means that their use for microtubule transport would be possible during interphase. However, the implications of using this protein/complex would become particularly apparent during cell division when the dynein/dynactin complex is also required to interact with the kinetochores. If the BTV replication mechanism did interact with such a cellular complex, it could also disrupt cell division and mitosis.

The rate of endothelial cell division would be low when they form a complete layer, as in a blood vessel (a target for BTV infection in the ruminant host). In the absence of cell division, the kinetochores will not be exposed to NS2 due to the presence of the nuclear membrane. The mitotic arrest phenomenon would therefore be expected to have little effect on the endothelial layer of blood vessels. It is possible that two separate phenomena are observed: one where NS1 disrupts the centrosome and causes multiple spindles, and another where NS2 blocks chromosome binding to the microtubules. These two processes may be linked, with the block of microtubule binding resulting in re-initiation of spindle formation.

It is difficult to speculate on the advantages of establishing a viral inclusion body at the kinetochore. This strategy is clearly not essential, as the kinetochores are only exposed during mitosis. The VIB may develop at this location, due to its tendency to multimerise and its interactions with components of the cytoskeleton, e.g. tubulin (Ross-Smith, 2008).

7.8: BTV NS1 and the centrosome

During this project a link was demonstrated between NS1 and the centrosome. NS1 was observed by electron microscopy, localised to the centrosomal region (P. Monaghan, personal communication). Confocal data in Chapters 4 and 6 showed that NS1, whether derived from the virus, or from a transfected expression plasmid, localised to a large mass immediately adjacent to the nucleus. Immunolabelling for centrosomal proteins confirmed co-labelling with NS1 localised to the centrosomal region. Immunolabelling also showed that the MTOC is disrupted during BTV infection, with proteins including centrin and FOP becoming more diffuse, or split into multiple fragments.

It would be interesting to express NS1 in isolation and explore the mechanism of MTOC disruption. Disruption of the centrosome has been reported previously for several viruses including African swine fever virus, tobacco mosaic virus and vaccinia virus (Ferralli et al., 2006; Jouvenet and Wileman, 2005; Ploubidou et al., 2000), but not previously by members of the *Reoviridae*.

The presence of multiple spindles was one of the most characteristic aspects of BHK-21 cells undergoing aberrant mitosis. It is unclear at this point why these multiple spindles arise. It is possible that the increase in spindle number is a result of NS1 causing the centrosome to divide, with the resulting MTOCs capable of supporting multiple spindle formation. Alternatively, multiple spindles may be derived by the cell making repeated attempts at mitosis having failed a specific 'checkpoint' due to their interaction with the chromosomes being blocked by NS2.

The restricted transcription associated with mitotic arrest for a prolonged period can cause apoptosis (Blagosklonny, 2007), as observed during this project. It may appear disadvantageous for the virus to induce cell death, if it does not replicate sufficiently fast to complete the cycle. However, a clear advantage to BTV of inducing mitotic arrest is the massive reduction in host cell transcription which occurs during mitosis (Blagosklonny, 2007). A suppression of host cell transcription essentially releases substrates for the virus to use, which would otherwise be utilised by the cell, e.g. NTPs. In relation to the host cell translation discussed above, the induction of mitotic arrest would add another level of inhibition of host cell macromolecule / protein synthesis. The nature of the viral replication strategy, involving the synthesis of mRNAs from a transcriptionally active core, combined with a translation mechanism that is clearly

distinct from the host cell translation, results in the creation of a cellular environment which becomes increasingly biased towards virus replication.

The impact of BTV infection in insect cells is unknown, as no work with insect cells was undertaken here. It would be disadvantageous to the midge, and in turn the virus, if the midge fitness suffered as a result of virus infection. It may therefore be important that the virus has little if any impact upon the insect cell cycle.

7.9: Bluetongue virus non-structural proteins

BTV non-structural proteins are intimately linked to the replication of the virus. This project represents an investigation into the role of non-structural proteins in the mammalian cells to determine more precisely the impact of NS1 and NS3/3A proteins on the outcome of infection. The cytopathogenicity of BTV infection in insect vs. mammalian cell culture systems is very different, and reflects the severity of infection observed in the whole animal.

This study has highlighted areas of similarity between BTV and other reoviruses, and has indicated several areas where the non-structural proteins play an important role in bluetongue virus replication.

7.10: Future Work

7.10.1: artificial expression of BTV proteins

The shut-off of protein synthesis in BTV-infected cells, including shut-off of expression from plasmids, highlights a need to reassess the experimental approach used to increase the level of a particular protein in the presence of BTV replication. The data obtained in suggest that it would be best to synthesise 'BTV-like' mRNAs, as opposed to 'mammalian-like' from the plasmid, to avoid shut-off. An internal ribosomal entry site (IRES) could be transcribed at the 5' end of the RNA. This would allow translation regardless of the cap-dependency of translation during BTV infection (Stirling 1996). This strategy would also allow the use of a simple T7 phage promoter and cell lines expressing the T7 phage polymerase, e.g. BSR T7/5 cells (Buchholz et al., 1999). A precise BTV 3' end to the RNA (to give the BTV 3' UTR) could be generated by inserting a hepatitis delta ribozyme immediately downstream of the UTR (Baron and Barrett, 1997). This would allow expression from BTV-like mRNAs, to enhance expression of NS1 or NS3, even in the presence of BTV infection.

An alternative approach that could be used to artificially enhance expression of specific virus protein(s) would be the generation an inducible cell line. Various systems exist for induction of protein expression after addition or removal of an effector molecule, e.g. tetracycline in the Tet-Off/Tet-On systems. This approach would give confidence that all cells contain the protein, eliminating background CPE from untransfected cells. The point at which expression of the protein occurs could also be controlled.

However, a potential issue with inducing protein expression after BTV infection has been established, is the possibility that non-BTV polymerases will be inhibited by the virus, (whether phage or mammalian). The transfection of *in vitro* synthesised BTV-like mRNAs may therefore represent the best approach for these studies.

Continued use of a siRNA approach to reducing the level of protein synthesis would build on the initial studies already established during this project. Firstly, the work could use of control siRNAs, as well as those synthesised in this project, to establish whether BTV possesses an anti-silencing mechanism. Secondly it would then be possible to further test the hypothesis by Owens *et al* (2004) in both mammalian and insect cells, that a high level of NS1 relative to NS3/3A is responsible for the CPE.

7.10.2: studies on BTV and siRNA

There is much work that can be performed to further elucidate the precise strategy that BTV uses to translate viral mRNAs. It would be of particular interest to determine the requirement for cellular initiation factors in BTV translation. This could be performed using established siRNAs (Blakqori *et al.*, 2009; Montero *et al.*, 2006).

7.10.3: BTV and host cell protein synthesis

A stark difference between the actions of RV NSP3 and BTV NS2, is the shut-off mechanism for poly(A) dependent translation that is caused by NSP3, but not by NS2. The data obtained in these studies clearly show a reduction in the translation of polyadenylated mRNAs during BTV infection, whilst the mechanism behind this inhibition remains unresolved. Earlier studies with BTV mRNAs have indicated shut-off of cap-dependent translation in BTV infected cells (Stirling 1996), suggesting that BTV may use a different mechanism to RV, to suppress host cell translation despite using a similar method to stimulate translation of its own mRNAs. Future studies in this area could usefully focus on the circularisation of the mRNA, and also a more in-depth analysis of PABP during infection.

It would be interesting to explore the evolutionary links and restraints between BTVs with regards to differing translational capacities. Reassortant BTVs have been observed where the virus has incorporated the NS2 gene from a different strain. It would be interesting to investigate the degree to which the NS2 genes of heterologous BTVs (or even closely related orbiviruses, or functional homologues from other reoviruses) can substitute for the NS2. It is unclear if a similar translation strategy, utilising NS2, is used by all of the BTV genome segments, as it has recently been suggested that NS1 regulates the translation of Seg-10 mRNA to produce NS3/3A (Mark Boyce, personal communication). Based upon the hypothesis that interplay between NS1 and NS3/3A determines cytopathogenicity, it would be interesting to see if NS1 controls NS3/3A expression, while NS2 may be required for efficient translation of the other nine proteins (including NS1).

7.10.4: cell division

One hypothesis resulting from data obtained in this project regarding the induction of multiple spindles, is that NS2 blocks the access of microtubules to the kinetochore due to an interaction with a microtubule end binding protein (as shown by confocal microscopy). Identification of the cellular protein/complex that interacts with the ‘plus end’ would be an important further step in investigation of this hypothesis. To explore this, a plasmid expressing BTV NS2 (pcDNA3.1V5/HIS_NS2, chapter 6) is being used by a collaborating group (Leeds Institute of Molecular Medicine) in an attempt to co-immunoprecipitate target proteins from uninfected cells.

The disruption of the centrosome by BTV infection is a novel and interesting observation. However, the mechanism of, or indeed the reason for, disrupting the centrosome remains unknown. An initial question would be whether the disruption is necessary for BTV replication, or is an by-product of another process. Clearly more work is required to determine the role of NS1 in BTV infection in both insect and mammalian cells. The reagents assembled, along with initial observations during this project, help lay a foundation for further work in this area.

The advent of BTV reverse genetics has given added significance to data concerning the role of non-structural proteins during BTV replication (Boyce et al., 2008; Roy et al., 2009). The BTV non-structural proteins represent good targets for artificial attenuation of the virus since, by definition, they are not required for the structure or antigenicity of the virus particle. It is conceivable that bluetongue viruses can be

manipulated, based on the data obtained in this project. The low level CPE seen in cells transfected with plasmids expressing NS3/3A (in Chapter 4) suggests some involvement in the pathogenicity observed in the ruminant host. A virus could be engineered where NS3/3A is altered/deleted, reducing or eliminating the impact of the protein. Similarly, if the specific mechanism behind inhibition of poly(A)-dependent translation observed in Chapter 5 is elucidated, it may be possible to alter the virus so that shut-off does not occur. A virus which does not shut off mammalian host cell protein synthesis might allow the host to mount a more effective immune response, without the clinical signs / damage associated with some of the monovalent live attenuated vaccines (Veronesi et al 2005. 2009).

While there remains much work to be performed in the mammalian system, virtually no work has been performed in the insect cell system which supports persistent infections (KC cells). This is clearly an area that demands further attention. The insect systems cannot be ignored and it is anticipated that future studies will increasingly incorporate insect related factors.

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Appendix I

Standard Solutions

Phosphate buffered saline (PBS)	10 mM Na ₂ HPO ₄ , 150 mM NaCl, pH 7.5
Luria Bertani (LB) Medium	1 % (w/v) Bacto-tryptone 0.5 % (w/v) Yeast extract 0.5 % (w/v) NaCl In deionized water
LB plates	LB medium + 1.5 % agar
TBS-Tween	50 mM Tris-HCL (pH 7.5) 150 mM NaCl 0.1 % (v/v) Tween-20
Tris-Glycine running buffer	200 mM glycine 25 mM Tris 0.1 % (w/v) SDS
Tris-Glycine transfer buffer	120 mM Tris 40 mM Glycine 20 % Methanol
Lysis buffer C	120 mM NaCl 50 mM Tris-HCL (pH 7.5) 0.5 % (v/v) Nonidet P-40 In deionized water
PBS/BSA blocking buffer	0.5 % (w/v) bovine serum albumin In phosphate buffered saline
PAGE Resolving gel (10 %)	10.64 % acrylamide 0.36 % bis-acrylamide 0.375 M Tris-HCL, pH 8.8 0.1 % SDS 0.05 % APS 0.0005 % TEMED
PAGE stacking gel	4 % acrylamide 0.13 % bis-acrylamide 0.125 M Tris-HCL, pH 6.8 0.1 % SDS 0.05 % APS 0.0005 % TEMED

Destain	7.5 % acetic acid 20 % methanol In deionized water
TFB1	30 mM KAc 50 mM MnCl ₂ 100 mM KCl 10 mM CaCl ₂ 15 % (v/v) glycerol Deionized water to a final volume of 50 ml
TFB2	10 mM Na-MOPS 75 mM CaCl ₂ 10 mM KCl 15 % (v/v) glycerol Deionized water to a final volume of 50 ml
4 % Paraformaldehyde	4 % in PBS, pH 7.5-8.0

Appendix II

Peer reviewed papers produced during the period of these studies.

Shaw, A.E., Reid, S.M., Knowles, N.J., Hutchings, G.H., Wilsden, G., Brocchi, E., Paton, D. and King, D.P. (2005). Sequence analysis of the 5' untranslated region of swine vesicular disease virus reveals block deletions between the end of the internal ribosomal entry site and the initiation codon. *Journal of General Virology* 86: 2753-2761.

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Appendix III

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Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1[☆]

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Abstract

Since 1998, multiple strains of bluetongue virus (BTV), belonging to six different serotypes (types 1, 2, 4, 8, 9 and 16) have caused outbreaks of disease in Europe, causing one of the largest epizootics of bluetongue ever recorded, with the deaths of >1.8 million animals (mainly sheep). The persistence and continuing spread of BTV in Europe and elsewhere highlights the importance of sensitive and reliable diagnostic assay systems that can be used to rapidly identify infected animals, helping to combat spread of the virus and disease.

BTV has a genome composed of 10 linear segments of dsRNA. We describe a real-time RT-PCR assay that targets the highly conserved genome segment 1 (encoding the viral polymerase—VP1) that can be used to detect all of the 24 serotypes, as well as geographic variants (different topotypes) within individual serotypes of BTV. After an initial evaluation using 132 BTV samples including representatives of all 24 BTV serotypes, this assay was used by the European Community Reference Laboratory (CRL) at IAH Pirbright to confirm the negative status of 2255 animals imported to the UK from regions that were considered to be at risk during the 2006 outbreak of BTV-8 in Northern Europe. All of these animals were also negative by competition ELISA to detect BTV specific antibodies and none of them developed clinical signs of infection. These studies have demonstrated the value of the assay for the rapid screening of field samples.

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Keywords: Bluetongue virus; Real-time RT-PCR; Diagnosis

1. Introduction

Bluetongue virus (BTV) is the type species of the genus *Orbivirus* within the family *Reoviridae*. The virus is transmitted by adult females of certain *Culicoides* species (biting midges) and can infect both ruminants and camelids (MacLachlan, 1994). Severe bluetongue disease (BT) is usually seen only in sheep and some species of deer. Clinical signs and significant levels of mortality are occasionally seen in infected cattle and goats. During the 2006 outbreak of BTV-8 in Northern Europe, ~10%

of cattle in affected areas developed clinical signs, and there was a case-fatality rate of up to 10% in these animals (~1% of the total cattle population) (EFSA, 2007).

BTV is widely distributed around the world, particularly in warmer climates (Purse et al., 2005), although the disease is primarily associated with its introduction (or at least introduction of a novel strain) to a new geographical area (Mellor and Wittmann, 2002). Historically Europe has experienced only sporadic incursions of BT, involving a single virus serotype on each occasion. However, since 1998, BT outbreaks have occurred annually, involving strains from six distinct BTV serotypes (1, 2, 4, 8, 9 and 16). Most of these types have persisted in the region for several years, resulting in the loss of over 1.8 million animals (Breard et al., 2004, 2005; Mellor and Wittmann, 2002).

Culicoides imicola is a major vector species involved in BTV transmission in Africa, Asia and southern Europe that has grad-

* GenBank accession numbers for novel sequences: EF059720 to EF059743.

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ually moved northwards into Europe, possibly in response to climate change in the region (Purse et al., 2005), which may have contributed to the increased incidence of BT in these areas. However, some of the recent European outbreaks (since 1998) have occurred beyond the distribution of *C. imicola*, suggesting the involvement of other vector species. Members of the *C. obsoletus* and *C. pulicaris* groups, are abundant across much of central and Northern Europe (including the UK) and have been implicated as BTV vectors (Caracappa et al., 2003; Carpenter et al., 2006; De Liberato et al., 2005; Purse et al., 2005; Savini et al., 2005). In August 2006 an outbreak of BT occurred in the Netherlands, Belgium, Germany, Luxembourg and north east France, 5 degrees further north within Europe than ever before, again confirming that BTV can be transmitted in the absence of *C. imicola*. Occurrence of the disease so far north suggests that the whole of Europe should now be considered at risk from BTV, and possibly from other arthropod-borne diseases.

BTV and the related orbiviruses can be transmitted over large distances, through trade (and movement) of infected host animals. This highlights a need for reliable assay systems to detect BTV infection and rapidly identify infected animals. Although cELISA can be used to detect BTV specific antibodies in serum samples (Anderson, 1984; Anderson et al., 1993), these antibodies are not generated until 7–10 days post-infection. The methods used to detect and serotype virus in blood can take several weeks, involving virus isolation (e.g. in embryonated chicken eggs), adaptation to cell culture and virus or serum neutralisation tests (VNT or SNT). These assays also depend on the availability of highly characterised antigens and antibodies for the cELISA (to identify the virus species/serogroup), and reference antisera or reference strains for all 24 BTV serotypes for the neutralisation assays (to identify virus serotype).

Assays based on reverse transcription polymerase chain reactions (RT-PCR), can be used to detect BTV RNA in clinical samples (e.g. blood or spleen) without virus isolation and do not require standardised serological reagents. However, many of the published RT-PCR based methods have not been fully evaluated for the detection of different BTV serotypes or topotypes and some published methods will only detect certain BTV strains (Aradaib et al., 2005, 1998, 2003; Billinis et al., 2001; Dangler et al., 1990; Jimenez-Clavero et al., 2006; Orru et al., 2004; Shad et al., 1997; Wade-Evans et al., 1990; Zientara et al., 2002). The majority of published primer sets target BTV genome segment 5 (Seg-5—coding for NS1), or genome segment 7 (Seg-7—coding for VP7) (Anthony et al., 2007; Aradaib et al., 2005, 2003; Jimenez-Clavero et al., 2006). Although these genome segments are relatively conserved across the BTV virus-species, they are sufficiently divergent between distinct orbiviruses to remain BTV specific. Indeed, VP7 is the major BTV serogroup-specific antigen (Gumm and Newman, 1982; Huismans, 1981). NS1 is also highly conserved (Huismans and Cloete, 1987) and Seg-5 is recommended as an RT-PCR target by the Office International des Epizooties (OIE) (OIE, 2004).

Real-time RT-PCR (rRT-PCR) assays offer certain advantages over traditional gel-based RT-PCR methods. The target amplicon is usually smaller, reducing the potential for problems caused by target degradation. Detection of a specific gene

sequence by rRT-PCR involves monitoring fluorescence generated by cleavage of a target specific oligonucleotide probe during amplification. This format eliminates the need to open the reaction tube post-amplification, for either a nested step or final gel analysis of the cDNA products, greatly reducing the risk of cross-contamination. The rRT-PCR format is therefore particularly well suited to diagnostic procedures where high reliability, throughput, sensitivity and specificity are required. However, real-time PCR probes can be very sensitive to probe-target mismatches and even relatively few base differences can potentially give false negative results (Jimenez-Clavero et al., 2006). This suggests that the more highly conserved regions of the BTV genome provide the most appropriate targets for development of BTV specific rRT-PCR assays. BTV Seg-1 encodes the viral polymerase, one of the most highly conserved proteins of all members of the family *Reoviridae* (Huang et al., 1995). However, Seg-1 also contains regions of sequence that remain BTV-specific.

We report the development of primers and probes for use in a real-time RT-PCR based assay, targeting BTV Seg-1. These reagents and methods were evaluated with a wide range of different BTV samples ($n=132$) derived from different geographic locations (different topotypes), including all 24 BTV serotypes, as well as representative strains of other closely related orbiviruses. The final 'combined' assay has been evaluated for use on a variety of test samples, including tissue culture derived virus, infected tissue and blood samples from cattle and sheep and infected adults of *Culicoides* vector insects. The assay has been used extensively to confirm the negative status of animals ($n=2255$) imported to the UK during 2006–2007. These animals were also negative by competition ELISA to detect BTV specific antibodies and none of them developed clinical signs of infection. These studies demonstrate the value of the assay for rapid screening of field samples.

2. Materials and methods

2.1. Virus samples

The BTV isolates used in this study are shown in Table 1. An isolate of the South African reference strain of BTV-1 (RSArrm/01) and a cell culture grown isolate of the BTV-16 vaccine strain (RSAvvvv/16) from the Orbivirus Reference Collection at the Institute for Animal Health (IAH) Pirbright (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/orbiviruses.htm) were used for the initial development of a Seg-1 specific assay. Virus samples in the collection are generally given an identification code which consists of a three letter abbreviation for the country of origin, the year of sampling and the cumulative isolate number for that country/year. A selection of other BTV isolates, representing each of the 24 serotypes (including viruses from different geographic origins), showing significant genetic variation (based upon phylogenetic analyses of VP2, Maan et al., 2007), were selected to validate the assay and assess its diagnostic sensitivity. Representative isolates of other *Orbivirus* species, including *African horse sickness virus* (AHSV), *epizootic haemorrhagic disease virus* (EHDV), *equine*

Table 1

Samples and bluetongue viruses tested as part of this study

IAH-Pirbright reference collection number	Origin/source	Serotype ^a	Mixed probe	Probe BTV-RSA 341–320P	Genbank accession number ^b
RSArrm/01	S. Africa	BTV-1	23.05	13.70	
GRE2001/01	Lesbos	BTV-1	17.23	20.15	
GRE2001/02	Lesbos	BTV-1	18.39	20.42	EF059720
IND1992/02	India	BTV-1	18.21	21.60	
IND2001/01	Chennai	BTV-1	22.27	23.80	
SUD1987/01	Sudan	BTV-1	14.34	15.08	
NIG1982/01	Nigeria	BTV-1	13.19	14.57	
AUS1981/01	Australia	BTV-1	16.04	17.61	
GRE2001/03	Greece	BTV-1	19.21		
GRE2001/04	Greece	BTV-1	16.31	17.10	
ALGERIA No3 (spleen) ^c	Algeria	BTV-1	18.23		
ALGERIA No10 (blood) ^c	Algeria	BTV-1	19.97		
ALGERIA No32/ov5 (blood) ^c	Algeria	BTV-1	24.32		
ALGERIA No6 (blood)	Algeria	Isolation – ve at CRL		24.11	
ALGERIA No7 (blood)	Algeria	Isolation – ve at CRL		23.07	
ALGERIA No21 (blood)	Algeria	Isolation – ve at CRL		27.00	
RSArrm/02	S. Africa	BTV-2	17.87	16.14	
ZIM2003/01	Zimbabwe	BTV-2	9.75	9.69	
ITL2002/07	Sicily	BTV-2	10.06	12.05	
ITL2002/06	Raccaja	BTV-2	14.36		
ITL2002/05	S. Angelo di Brolo	BTV-2	9.73	9.39	
ITL2002/04	S. Pietro Patti	BTV-2	7.87		
ITL2002/03	Novara di Sicilia	BTV-2	13.09		
ITL2002/02	Novara di Sicilia	BTV-2	12.94		
ITL2002/01	Novara di Sicilia	BTV-2	17.67		
IND1982/01	India	BTV-2	16.11		
NIG1982/02	Nigeria	BTV-2	19.5		
FRA2001/03	Corsica	BTV-2	23.88		
FRA2001/06	Corsica	BTV-2	11.92	11.05	
TUN2000/01	Tunisia	BTV-2	16.31	21.18	
SAD2001/02	Sardinia	BTV-2	18.61	16.94	
SAD2002/01	Sardinia	BTV-2	21.46		
SAD2001/04	Sardinia	BTV-2	16.58		
SAD2002/03	Sardinia	BTV-2	17.8		
SAD2001/06	Sardinia	BTV-2	20.21		
RSArrm/03	S. Africa	BTV-3	16.04	13.78	
ZIM2002/01	Zimbabwe	BTV-3	18.37	18.12	
ZIM2002/02	Zimbabwe	BTV-3	21.65	22.63	
NIG1982/06	Nigeria	BTV-3	18.39	20.71	EF059721
RSArrm/04	S. Africa	BTV-4	17.62	15.28	
RSAvvv3/04	S. Africa	BTV-4	21.53	22.44	
RSAvvv5/04	S. Africa	BTV-4	23.91		
RSAvvv7/04	S. Africa	BTV-4	20.52	21.63	
TURvvvv/04	Turkey	BTV-4	11.42	11.33	
GRE1999/02	Lesbos	BTV-4	15.96		
GRE1999/03	Lesbos	BTV-4	15.6	13.81	
GRE1999/05	Pieria	BTV-4	16.5		
TUR1978/01	Turkey	BTV-4	15.03	15.10	
SUD1983/01	Sudan	BTV-4	17.46		
GRE2000/03	Greece	BTV-4	20.48		
GRE2000/06	Greece	BTV-4	23.55		
ARG2002/01	Argentina	BTV-4	14.33	14.77	EF059722
ARG2002/02	Argentina	BTV-4	15.79		
ARG2002/03	Argentina	BTV-4	16.54	17.11	
ARG2002/04	Argentina	BTV-4	18.22		
SPAIN 1 (blood) ^c	Spain	BTV-2	28.41		
SPAIN 2 (blood) ^c	Spain	BTV-4	24.21		
SPAIN 3 (blood) ^c	Spain	BTV-4	25.98	22.65	
SPAIN 4 (blood) ^c	Spain	BTV-4	26.39	23.44	
SPAIN 5 (blood) ^c	Spain	BTV-4	25.02	22.47	EF059723
SPAIN 6 (blood)	Spain	Isolation – ve at CRL		27.16	23.32
GRE1999/10	Greece	BTV-4	20.75		
GRE1999/11	Greece	BTV-4	19.07		

Table 1 (Continued)

IAH-Pirbright reference collection number	Origin/source	Serotype ^a	Mixed probe	Probe BTV-RSA 341–320P	Genbank accession number ^b
RSArm/05	S. Africa	BTV-5	18.12	14.61	
NIG1982/03	Nigeria	BTV-5	17.92		
NIG1982/04	Nigeria	BTV-5	16.71	18.89	EF059724
CAR1982/02	Cameroon	BTV-5	18.49	20.51	
RSArm/06	S. Africa	BTV-6	16.29	14.25	EF059725
RSArm/07	S. Africa	BTV-7	18.49	19.25	EF059726
RSArm/08	S. Africa	BTV-8	20.75	16.63	EF059727
NIG1982/07	Nigeria	BTV-8	16.16		
KEN—/01	Kenya	BTV-8	17.55	No Ct	
NET2006/01 (blood)	Netherlands	BTV-8	20.35		
RSArm/09	S. Africa	BTV-9	16.65	15.90	
RSAvvv1/09	S. Africa	BTV-9	25.25	No Ct	EF059728
RSAvvv2/09 ^d	S. Africa	BTV-9	24.00 [No Ct]		
RSAvvv9/09	S. Africa	BTV-9	18.78		
ITL2003/01 ^d	Sicily	BTV-9	20.88 [No Ct]	No Ct	
GRE1999/06	Halkidiki	BTV-9	9.82	7.07	
TUR2000/11	Turkey	BTV-9	8.37		
TUR2000/04	Turkey	BTV-9	20.17		
TUR2000/05	Manisa	BTV-9	21.23		
TUR2000/03	Manisa	BTV-9	21.34		
BUL1999/01	Bulgaria	BTV-9	18.14	18.34	
GRE2000/02	Greece	BTV-9	6.54		
BOS2002/01	Vlasenica	BTV-9	19.03	18.52	
BOS2002/02	Vlasenica	BTV-9	18.7		
BOS2002/03	Vlasenica	BTV-9	16.42		
BOS2002/04	Vlasenica	BTV-9	17.39		
KOS2001/01	Kosovo	BTV-9	16.85		
KOS2001/02	Kosovo	BTV-9	18.18		
KOS2001/03	Kosovo	BTV-9	17.4		
KOS2001/04	Kosovo	BTV-9	16.31		EF059729
KOS2001/04 ^e (culicoides)	Kosovo	BTV-9	19.3	20.57	
KOS2001/04 ^e (culicoides)	Kosovo	BTV-9	22.7	22.33	
KOS2001/04 ^e (culicoides)	Kosovo	BTV-9	25.07	26.18	
KOS2001/04 ^e (culicoides)	Kosovo	BTV-9	26.36	27.03	
RSArm/10	S. Africa	BTV-10	15.52	15.62	
RSArm/11	S. Africa	BTV-11	16.25	15.06	
ZIM2003/05	Zimbabwe	BTV-11	9.04	11.70	
NIG1982/08	Nigeria	BTV-11	16.29	16.67	
ZIM2003/02	Zimbabwe	BTV-11	11.21		
ZIM2003/03	Zimbabwe	BTV-11	10.25		
RSArm/12	S. Africa	BTV-12	16.93	14.50	
ZIM2003/04	Zimbabwe	BTV-12	10.34		EF059730
NIG1982/09	Nigeria	BTV-12	18.18	No Ct	
KEN—/—	Kenya	BTV-12	18.65		
RSArm/13	S. Africa	BTV-13	17.08	17.44	
RSArm/14	S. Africa	BTV-14	15.34	15.02	
CAR1982/04	Cameroon	BTV-14	15.91	16.78	EF059731
RSArm/15	S. Africa	BTV-15	17.21	15.76	
ZIM2003/08	Zimbabwe	BTV-15	9.64	6.93	EF059732
ZIM2003/09	Zimbabwe	BTV-15	8.00		
RSArm/16	S. Africa	BTV-16	27.19		EF059733
TUR2000/09	Izmir	BTV-16	9.77		
TUR2000/08	Manisa	BTV-16	9.26		
TUR2000/01	Izmir	BTV-16	19.1	20.58	
TUR2000/02	Manisa	BTV-16	19.15		
TUR2000/10	Izmir	BTV-16	20.19		
NIG1982/10	Nigeria	BTV-16	18.55	20.10	
RSAvvv/16	S. Africa	BTV-16	23.88	22.07	EF059734
CYP2004/001	Cyprus	BTV-16	15.82	18.19	
RSArm/17	S. Africa	BTV-17	14.31	13.17	
RSArm/18	S. Africa	BTV-18	15.34	16.71	EF059735
RSArm/19	S. Africa	BTV-19	17.74	16.03	EF059736
RSArm/20	S. Africa	BTV-20	18.17	16.53	EF059737
AUS1978/01	Australia	BTV-20	21.45	21.21	

Table 1 (Continued)

IAH-Pirbright reference collection number	Origin/source	Serotype ^a	Mixed probe	Probe BTV-RSA 341–320P	Genbank accession number ^b
RSArr/21	S. Africa	BTV-21	22.03	21.15	EF059738
RSArr/22	S. Africa	BTV-22	18.24	17.27	
NIG1982/11	Nigeria	BTV-22	18.14	19.80	EF059739
RSArr/23	S. Africa	BTV-23	16.5	14.37	
IND1998/01	Bangalore	BTV-23	15.76		
IND1988/02	Rahuri	BTV-23	16.46	19.27	
IND1997/01	Bangalore	BTV-23	16.08		
IND1988/03	Rishikesh	BTV-23	13.19		EF059740
RSArr/24	S. Africa	BTV-24	15.4	14.13	EF059741

Ct values obtained using either probe RSA-BTV 341–320 alone or both probes incorporated into a single reaction. In every case both primer sets RSA and UNI were included in the reaction. Viruses sequenced as part of the study are indicated.

^a BTV: bluetongue virus.

^b GenBank accession numbers of the isolates sequenced as part of this study.

^c BTV was successfully isolated from these clinical samples. More information about individual virus samples (catalogued by their IAH-Pirbright Reference collection number) is available at: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/orbiviruses.htm.

^d The sample preparations for these samples required dilution to be efficiently detected.

^e RNA isolated from individually homogenised *Culicoides* midges infected with BTV-9.

encephalitis virus (EEV), *Wad Medani virus* (WMV), *Chobart Gorge virus* (CHV) and samples of unassigned orbiviruses (Table 2) were used to test assay specificity. To ensure that there is no cross reaction with the virus host species, nucleic acid samples extracted from uninfected EDTA treated ovine and bovine blood of UK origin (from 6 sheep and 10 cattle), as well as three homogenised colony-bred *Culicoides*, were tested using the optimised assay (Table 2).

Six EDTA treated blood samples, taken from cattle in Spain during the 2005 BTV outbreak (and successfully used for virus isolation at Laboratorio Central de Veterinaria, Algete, Spain), were tested for the presence of BTV RNA by rRT-PCR and virus isolation at IAH Pirbright (Table 1). Six samples of EDTA treated sheep blood taken at the start of the BTV-8 outbreak in the Netherlands during August 2006, that had been tested for the presence of BTV specific antibodies by cELISA (Anderson,

Table 2

A variety of non-BTV orbiviruses and host species nucleic acid were tested in order to confirm specificity for BTV nucleic acid

IAH-Pirbright reference collection number	Origin/source	Serotype ^a	Mixed probe	Probe BTV-RSA 341–320P	GenBank accession number ^b
RSArrah/01	S. Africa	AHSV-1	No Ct	No Ct	
RSArrah/02	S. Africa	AHSV-2	No Ct	No Ct	
RSArrah/03	S. Africa	AHSV-3	No Ct	No Ct	
RSArrah/04	S. Africa	AHSV-4	No Ct	No Ct	
RSArrah/05	S. Africa	AHSV-5	No Ct	No Ct	
RSArrah/06	S. Africa	AHSV-6	No Ct	No Ct	
KENrrah/07	S. Africa	AHSV-7	No Ct		
PAKrrah/09	S. Africa	AHSV-9	No Ct	No Ct	
Bryanstone	—	EEV-1	No Ct	No Ct	
Cascara	—	EEV-2	No Ct	No Ct	
Kaaplas	—	EEV-4	No Ct	No Ct	
Kyalami	—	EEV-5	No Ct	No Ct	
Potchefstroom	—	EEV-6	No Ct	No Ct	
AUS1995/02	—	EHDV-1	No Ct	No Ct	EF059742
USA—/01	—	EHDV-1	No Ct	No Ct	EF059743
Andasibe virus	—	Orbivirus	No Ct	No Ct	
Chobart Gorge virus	—	Orbivirus	No Ct	No Ct	
Japanaut virus	—	Orbivirus	No Ct	No Ct	
Matucare virus	—	Orbivirus	No Ct	No Ct	
Tembe virus	—	Orbivirus	No Ct	No Ct	
Tracambe virus	—	Orbivirus	No Ct	No Ct	
Wad Medani virus	—	Orbivirus	No Ct	No Ct	
Uninfected ovine blood	—	—	No Ct	No Ct	
Uninfected bovine blood	—	—	No Ct	No Ct	
Culicoides	—	—	No Ct	No Ct	

Ct values obtained using either probe RSA-BTV 341–320 alone or both probes incorporated into a single reaction. In every case both primer sets RSA and UNI were included in the reaction. Viruses sequenced as part of the study are indicated.

^a AHSV: African horse sickness virus; EEV: equine encephalitis virus; EHDV: epizootic haemorrhagic disease virus.

^b GenBank accession numbers of the isolates sequenced as part of this study.

1984) and for viral RNA using a conventional RT-PCR targeting Seg-7 (Anthony et al., 2007) were also tested using the rRT-PCR targeting Seg-1. These samples also generated BTV isolates NET2006/01 to NET2006/04. Six samples of EDTA treated sheep blood taken in Algeria, submitted to the CRL in 2006, were also tested for the presence of BTV RNA using the rRT-PCR assay.

After the initial evaluation of the assay it was subsequently used (during 2006–2007) to screen 2255 blood samples for the presence of the virus, to confirm the uninfected status of animals imported to the United Kingdom from affected countries in Northern Europe.

Samples of washed and sonicated sheep blood infected with the BTV-16 vaccine (RSAvvvv/16) which had been kept at +4 °C for over a year, were also tested using the robotic RNA extraction method.

The assay was also evaluated for detection of BTV in *Culicoides* midges. Colony-bred adult *C. sonorensis* were membrane-fed on infected blood (containing BTV-9 (KOS2001/04)), and maintained for 7–10 days at 25 °C to allow virogenesis, as previously described (Wittmann et al., 2002). Each midge was subsequently homogenised in 1 ml of Eagles tissue culture medium containing 0.6% antibiotics. In each case cell debris was removed by centrifugation (2 × 3 min at 12,000 RPM), producing a clarified tissue supernatant.

2.2. Sample preparation and RNA isolation

2.2.1. Spin column RNA purification

RNA was isolated from EDTA treated blood, clarified tissue/homogenised insect supernatant, or supernatant of cell cultures infected with specific BTV isolates (Table 1). In each case 140 µl of sample and the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in 50 µl nuclease-free water and then stored at –20 °C.

2.2.2. Robotic RNA purification

For the automated 'robotic' extraction of nucleic acid, 200 µl of sample was first added to 300 µl of Roche lysis/binding buffer. The resulting 500 µl was extracted on a Roche MagNA Pure LC robot using the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche, Mannheim, Germany) using the Total NA

External lysis.blk automated extraction programme, the nucleic acid being eluted in 50 µl elution buffer (MagNA Pure LC Total Nucleic Acid Isolation kit, Roche). This protocol was used to extract nucleic acid from EDTA treated blood samples from the 2006 outbreak of BTV-8 in northern Europe. These included samples from an experimental infection of cattle and sheep, conducted at the CRL, IAH Pirbright using the 2006 Netherlands BTV-8 strain (NET2006/01).

2.3. RNA denaturation

Two methods (heating and treatment with methyl-mercuric hydroxide (MMOH)) were evaluated for RNA sample denaturation prior to the initial RT step. For chemical denaturation, 2.5 µl samples of RNA were incubated with 2.5 µl 20 mM MMOH for 10 min at room temperature, followed by the addition of 1 µl 0.7 M β-2-mercaptoethanol (β-ME). The resulting 6 µl samples were added to 19 µl of the RT-PCR reaction mix. Heat denaturation of RNA was evaluated by heating 6 µl samples at 98 °C for 5 min (in a PCR reaction plate, using a thermal cycler) followed by rapid cooling on ice. The 19 µl of rRT-PCR mastermix was then added prior to incubation of the reactions. The initial work with different virus strains was carried out using RNA denaturation with MMOH. However, both methods were tested in sensitivity experiments and heat denaturation was shown to be equally effective. Field samples were subsequently denatured by heating, a method that is more suitable for automated systems (robotics).

2.4. RT-PCR and sequencing of Seg-1

Seg-1 sequences from representative isolates of BTV (AY493686, L20445, L20446, L20447, L20508, AY154458, X12819, NC_006023), African horse sickness virus (AHSV, NC_007656) and Epizootic haemorrhagic disease virus (EHDV, AB186040) were obtained from the GenBank database. These sequences were aligned using MegAlign (DNAStar, Lasergene) and used to design redundant primer sets (ORBI-UNI-F [nt 19–35] and ORBI-UNI-R [nt 430–414]—Table 3) to amplify Seg-1 from any of these *Orbivirus* species. These primers were used with the Qiagen OneStep RT-PCR kit, to amplify a 412 nucleotide fragment from near the 5' end of BTV or EHDV

Table 3

Primer and probe sequences used for RT-PCR, sequencing and real-time RT-PCR assays for the detection of BTV

Purpose	Oligo name	Sequence (5'-3')	Genomic location ^a
BTV SEG1 RSA (east)	Forward primer	GCGTTCGAAGTTACATCAAT	291–311
	Reverse primer	CAGTCATCTCTAGACACTCTATAATTACG	387–357
BTV SEG1 UNI (west)	Forward primer	GCTTTGAGCTGTACGTGAAC	291–311
	Reverse primer	TCTCCCTTGAAACTCTATAATTACG	381–357
BTV SEG1 probes	Probe RSA	CGGATCAAGTTCACTCCACGGT	341–320
	Probe 323	TCCCTCCGATCAAGTTCACTCCAC	346–323
RT-PCR + sequencing	Forward primer	YMATCACCGTCCAAGGT	19–35
	Reverse primer	TCCATYTCGTTTTMGC	430–414

BTB-UNI-F and BTB-UNI-R were used for the RT-PCR and sequencing of a 412 nt fragment of BTV Seg-1 from which the real-time RT-PCR primers and probes were designed. Combining the RSA (east) and UNI (west) sets of primers with both probes in a single assay allows pan-detection of BTV. Separate reactions using only primer set RSA or primer set UNI with both probes allows east or west viruses (respectively) to be identified and distinguished.

^a Genome location according to GenBank accession number AY154458.

Seg-1. The RT-PCR reactions contained 10 µl of 5× mastermix (Qiagen), 30 pmol of each primer, 10 mM of each dNTP, 2 µl of enzyme mix, and water to a final volume of 44 µl, before adding RNA that had been chemically denatured as described above. Amplification (Eppendorf mastercycler) conditions were 45 °C for 30 min (reverse transcription), 94 °C for 15 min (RT inactivation/hotstart), and 40 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min. Chain elongation was extended to 7 min for the final cycle. The cDNA products were visualised on a 1.2% agarose gel stained with ethidium bromide. Amplicons of the expected size were excised from the gel and purified using the QIAquick gel-extraction kit (Qiagen). Approximately 50 ng of the PCR product was used for direct sequencing of both strands by the chain termination method using the original PCR primers according to the manufacturer's instructions (Beckman Coulter, CEQ 8000 DNA analysis system). Raw sequence data for each isolate was assembled using SeqMan (DNAStar, Lasergene) and consensus sequences aligned using MegAlign (DNAStar, Lasergene).

2.5. Assay sensitivity

A 412 nt cDNA fragment from Seg-1 of RSAvvvv/16, was cloned into pGEM T-easy (Promega) according to the manufacturer's instructions. The identity of the insert was confirmed by sequencing. Positive strand RNA was transcribed using the T7 MEGAscript® High Yield Transcription Kit (Ambion). Transcribed RNA was DNase treated, purified using an RNA clean-up protocol (RNeasy, Qiagen) and extracted using Trizol LS (Invitrogen) according to the manufacturer's instructions. The purified RNA was quantified using the Bioanalyzer (Agilent) and a log-10 dilution series was generated to test the sensitivity limits of the assay.

2.6. BTV specific real-time RT-PCR primer and probe design

Based on the sequence data generated, primer sites were chosen in Seg-1 so as to cross-react with all BTV isolates, but not with other orbiviruses. There are two consensus sequences for each primer site (representing eastern and western genotypes) and therefore two sets of primers were designed. These primers (Table 3) have different lengths to balance primer Tms, and can be used to amplify a 97 bp fragment (eastern PCR) or 91 bp (western PCR).

Probes for the rRT-PCR reactions were designed according to standard TaqMan® specifications, based on a sequence alignment for different BTV isolates/serotypes. The probes (purchased from Sigma-Genosys) were labelled with 6-carboxyfluorescein (FAM) at the 5' end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3' end.

2.7. Real-time RT-PCR

PCR assays using the eastern (RSA) and western (UNI) genotype-specific primer sets (Table 3) were optimised separately. The two sets of primers were subsequently included in a

single combined reaction to allow the detection of either eastern or western viruses in a single tube reaction. The Superscript III/Platinum *Taq* one-step RT-PCR kit (Invitrogen) was used for all rRT-PCR assays. Each 19 µl of reaction mix contained the following: 12.5 µl 2× reaction buffer mix (kit), 20 pmol of each primer, 2.5 pmol each probe, 0.5 µl Mg₂SO₄ (kit), 0.5 µl diluted ROX reference dye (kit) and 0.5 µl of the Superscript III/Platinum *Taq* enzyme mix (kit). When only single probe and primer sets were included, the remaining volume was substituted with nuclease free water. Mastermix was distributed to the wells of Stratagene Mx3005 reaction plates. Mastermix preparation and distribution into wells was performed in a dedicated PCR hood in a different laboratory from the RT-PCR assembly hood. The 6 µl of denatured RNA was added to the reaction mix and the reaction capped using optical caps (Stratagene). Amplification was carried out in an Mx3005P PCR machine (Stratagene) using the following programme: 55 °C for 30 min, 1 cycle (reverse transcription), 95 °C for 10 min, 1 cycle (denaturation of the Superscript III and activation of the Platinum *Taq* DNA polymerase) and 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence was measured at the end of the 60 °C annealing/extension step. Cycle threshold (C_t) values for each sample were determined from the point at which fluorescence breached a threshold fluorescence line. Negative results are described as 'No C_t'.

2.8. BTV titration series

To determine PCR efficiency, a 10-fold serial dilution of BTV-4 (RSArrrr/04) cell culture supernatant was made in EDTA treated blood previously shown to be negative by rRT-PCR. This was extracted using the robotic methods described above and screened for BTV RNA using the optimised rRT-PCR assay. During these sensitivity studies, samples were assayed in duplicate using both chemical and heat denaturation methods. PCR efficiency was calculated using the Stratagene software (Mx3005P).

3. Results

3.1. Sequence comparison of genome segment 1

Comparisons of the Seg-1 sequences from different BTV strains (as listed in Table 1), identified a highly conserved 97 bp region, at nt 291–387 (numbered according to GenBank accession number AY154458). However, consensus sequences for this region were divided into two distinct groups, corresponding to BTV strains from eastern and western origins (with 78.3–87.6% identity between groups). The virus isolates in the eastern group (from the Middle-east, Asia and Australasia) showed 87.6–96.9% identity to a published sequence for Seg-1 from a Taiwanese isolate (AY493686, unknown serotype). The isolates in the western group (originating from Africa and the Americas—BTV-11 (L20445), BTV-13 (L20446), BTV-17 (L20447), BTV-2 (L20508), BTV-10 (NC_006023 and X12819)) showed a high level of similarity to BTV-2 from Corsica (92.8–97.9% identity relative to AY154458).

3.2. Development of a BTV specific real-time RT-PCR assay

Potential target footprints for development of RT-PCR primers (nt 291–311 and nt 381–357—Table 3) were identified within the conserved region of Seg-1. Both primer sites have two consensus sequences (representing eastern and western BTV genotypes) and two distinct sets of primers were therefore designed to amplify sequences from the different groups. A highly conserved sequence (nt 341–323) was also selected as the target for a TaqMan® probe (identified as RSA-BTV 341–320 in Table 3). Sequence comparisons indicated that these target sequences are significantly different from those of the same regions from the closely related *Orbivirus* species AHSV and EHDV (Fig. 1). The expected products amplified using the two primer sets are 97 bp (eastern PCR) or 91 bp (western PCR)

(Fig. 1). Using these primers ‘eastern-specific’ and ‘western-specific’ assays can be run separately but in parallel, or combined into a single reaction, to allow the ‘pan’ – detection of BTV strains from either group.

A selection of genetically and geographically distinct isolates of BTV, including isolates of all 24 BTV serotypes were initially tested to evaluate the ability of the assay to detect a diverse range of BTV strains (as indicated in Table 1). Although a ‘No Ct’ result was initially obtained with RNA from three isolates (RSAvvv1/09 [BTV-9], ITL2003/01 [BTV-9] and NIG1982/09 [BTV-12]), electrophoretic analysis of the reaction products showed successful amplification of the target region of Seg-1 from each of these samples. Subsequent sequencing of the amplicons generated identified mismatches between the probe and its target footprint for all three isolates (Fig. 1), indicating

(A)	BTVuni 291-311F	Probe RSA		Probe 323		BTVuni 381-357R
		GCTTTGAGGTGACGTGAGC	TGGCACTTGAACCTAGGCTCT	G.....	A.....	
BTW-2 (from AY154458)	G.....	A.....
BTW-2 (from L2050R)	G.....
BTW-3 [NIG1982/06 (EF059721)]	G.....
BTW-4 [ARG2002/01 (EF059722)]	G.....
BTW-4 [ISPA2005/05 (EF059723)]	G.....
BTW-5 [NIG1982/04 (EF059724)]	G.....
BTW-6 [RSArrrr/06 (EF059725)]	G.....
BTW-7 [RSArrrr/07 (EF059726)]	G.....
BTW-8 [RSArrrr/08 (EF059727)]	G.....
BTW-8 [NET 2006/04	G.....
BTW-9 [RSAvvv1/09 (EF059728)]	G.....
BTW-9 [RSAvvv2/09]	G.....
BTW-10 (from NC_006023)	G.....
BTW-10 (from X12819)	G.....
BTW-11 (from L20445)	G.....
BTW-12 [ZIM2003/04 (EF059730)]	G.....
BTW-12 [NIG1982/09]	G.....
BTW-13 (from L20446)	G.....
BTW-14 [CAR1982/04 (EF059731)]	G.....
BTW-15 [ZIM2003/08 (EF059732)]	G.....
BTW-17 (from L20447)	G.....
BTW-18 [RSArrrr/18 (EF059735)]	G.....
BTW-19 [RSArrrr/19 (EF059736)]	C.....
BTW-22 [NIG1982/11 (EF059739)]	T.....	G.....
BTW-24 [RSArrrr/24 (EF059741)]	G.....	G.....
BTW-16 [RSAvvvv/16 (EF059734)]	G.....
AHSV-9 (from NC_006021)
AHSV-9 (from U94887)
EHDV-1 [AUS1995/02 (EF059742)]
EHDV-1 [USA----/01 (EF059743)]
IBARAKI (from AB186040)

(B)	BTVrsa 291-311F	Probe RSA		Probe 323		BTVrsa 387-357R
		CGGTTCGAAGTTTACATCAAT	CGATTAATCTCACAGATCTCTACTGAC	
BTW-1 [GRE2001/02 (EF059720)]
BTW TAIWAN (from AY493686)
BTW-9 [KOS2001/04 (EF059729)]
BTW-16 [RSArrrr/16 (EF059733)]
BTW-16 [RSAvvvv/16 (EF059734)]
BTW-20 [RSArrrr/20 (EF059737)]
BTW-21 [RSArrrr/21 (EF059738)]
BTW-23 [IND1988/03 (EF059740)]
BTW-7 [RSArrrr/07 (EF059726)]
AHSV-9 (from NC_006021)
AHSV-9 (from U94887)
EHDV-1 [AUS1995/02 (EF059742)]
EHDV-1 [USA----/01 (EF059743)]
IBARAKI (from AB186040)

Fig. 1. Sequence alignment of the real-time RT-PCR primer and probe footprints for genome segment 1 of: BTV (serotypes 1–24), EHDV (serotype 1, eastern and western strains and Ibaraki virus), AHSV (serotype 9). (A) Represents viruses of western origin aligned against primer set UNI, but includes BTV-16 (RSAvvv/16) as a representative eastern virus. (B) Represents viruses of eastern origin aligned against primer set RSA, but includes BTV-7 (RSArrrr/07) as a representative western virus. Probe footprints for (A) and (B) are both aligned against probes RSA and 323.

that this was the most likely cause of the negative ('No Ct') results. A second probe (BTV 346–323 – moved three bases downstream and extended by a further two bases at its 3' end – Fig. 1) was therefore designed to match the same region of Seg-1 and improve detection of these three isolates (Table 1). When this second probe was tested in a combined assay (along with the original probe and both primer sets) it gave reliable detection of RSAvvv1/09 and NIG1982/09 with no reduction in assay performance.

3.3. Assay specificity

A more extensive evaluation of the combined assay (containing both probes and both primer sets) gave amplification with all of the BTV samples tested ($n=132$) (Table 1). However, inconsistent fluorescence results occurred with two isolates, both of BTV-9. These were the other sample of the South African BTV-9 vaccine (RSAvvv2/09) and the same Italian isolate of BTV-9 (ITL2003/01) that gave problems in the initial tests. Although an increasing fluorescence signal was obtained under standard test conditions with both RNA samples, it was not logarithmic. However, when the test samples were diluted 10-fold in nucleic acid free water, a strong positive result was consistently obtained in both cases, with Ct values of 24.00 (RSAvvv2/09) and 20.88 (ITL2003/01). In each case analysis of the standard reaction products by agarose gel electrophoresis, demonstrated the synthesis of an amplicon of the expected size even under standard conditions, suggesting that the primers were working effectively but poor probe binding to the template, was causing inconsistent results. As RSAvvv2/09 (not detected) has an identical sequence to RSAvvv1/09 (detected), the cause of the initial No Ct results was not thought to be sequence based.

Tests using the 'combined assay' with samples containing RNA templates from isolates of five other *Orbivirus* species (EHDV, AHSV, EEV, WMV, CGV) or five unassigned orbiviruses (Table 2), consistently gave 'No Ct' values. Similarly, no amplification was evident in samples containing only *Culicoides*, ovine or bovine nucleic acid (Table 2).

BTV RNA was also detected successfully in a variety of sample types, including infected cattle or sheep blood collected in EDTA during outbreaks in Spain (2005), Algeria (2006) and the Netherlands (2006), respectively, homogenised spleen from an Algerian sheep infected with BTV-1 in 2006, *Culicoides sonorensis* midges, cell culture supernatants, and BTV-16 infected sheep blood that had been washed, sonicated and stored at 4 °C for over a year. Virus was not recovered from one of the Spanish blood samples submitted to the CRL in 2005, despite virus being isolated from all six in Spain and all six giving positive results in the pan-BTV rRT-PCR assay. Similarly, virus was not isolated from three Algerian blood samples that were positive for BTV RNA using the pan-BTV rRT-PCR.

3.4. Assay sensitivity

A log-10 titration series of *in vitro* transcribed RNA indicated that fewer than 10 copies of BTV Seg-1 ssRNA could be detected. A log-10 titration series in blood of RSArrrr/04 cell

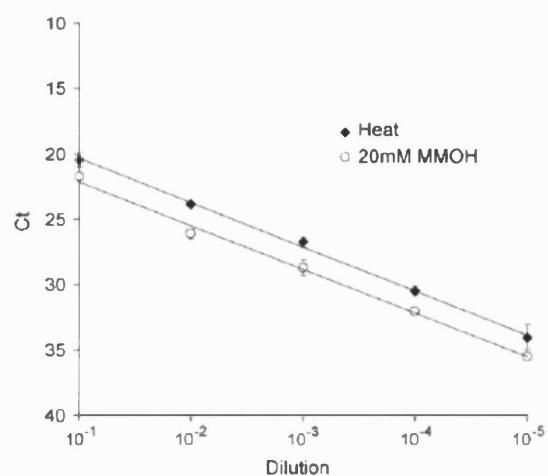


Fig. 2. Titration curves comparing denaturation of RNA samples by 20 mM methyl mercuric hydroxide (MMOH), or by heating for 5 min at 98 °C and then rapid cooling on ice. The Seg-1 rRT-PCR efficiencies were almost identical using these methods (MMOH 98.6%/heating 97.8%) but heat denaturation resulted in Ct values that were approximately two cycles lower. Bars represent standard deviation calculated from duplicate samples.

culture derived virus (tissue culture supernatant) showed that the viral RNA was detected down to a dilution of 10⁻⁵ (Fig. 2). It was found that heat denaturation gave lower Ct values (by approximately 2 Cts) indicating that it was slightly more efficient for denaturation of RNA templates. Duplicate reactions of a 10-fold titration of BTV-4 infected blood, using chemical denaturation methods, produced standard deviations ranging from 0.18 to 0.62, while heat denaturation gave standard deviations of between 0.03 and 0.94. Both methods of denaturation gave PCR efficiencies close to 100% (98.6% with MMOH, 97.8% with heat). Inter- ($n=3$ plates) and intra- ($n=2$ –4 replicates) plate reproducibility of the pan-BTV assay was tested using replicate samples of RSAvvv1/09. The standard deviation across all three independent plates was 1.19 Ct. Standard deviation for intra-plate variation ranged between 1 and 0.06 Ct.

By running assays containing both probes but individual rather than combined primer sets (RSA or UNI) in parallel with the pan-BTV assay (containing both primer sets and both probes), it was possible to detect and distinguish viruses from eastern or western origins. In each case the homologous primer set gave Ct values similar to those achieved using the combined pan-BTV assay (Fig. 3), while the assay containing the heterologous primer set gives a Ct value that was at least 10 cycles higher, or gave a 'No Ct' value.

4. Discussion

All 132 BTV samples tested using the combined assay gave positive Ct results and amplification plots. These included field strains of BTV serotypes 1, 9, 16 (all eastern genotype) and 2, 4, 8 (all western genotype) that were recovered since 1998 from European disease outbreaks. Only two strains of BTV gave inconsistent results. These were one of the two samples of the South African BTV-9 vaccine (RSAvvv2/09), and

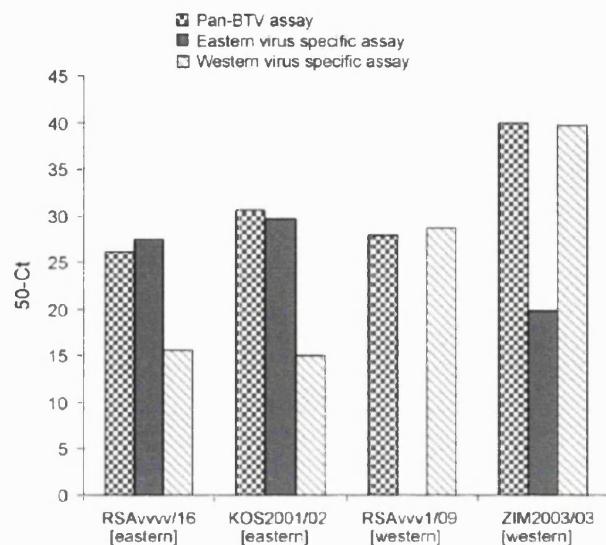


Fig. 3. By including only one Seg-1 primer set (RSA or UNI) it is possible to distinguish viruses of eastern origin from those of western origin. The appropriate primer set gives Ct values similar to those of the pan-BTV assay. The data shown includes two serotype 9 viruses (KOS2001/02 [east] and RSAvvv1/09 [west]), an eastern vaccine strain of BTV-16 (RSAvvv/16) and a western (African) strain of BTV-11 (ZIM2003/11). The eastern and western strains (even those of the same serotype) could be distinguished using this approach. Indeed, No Ct value was obtained for RSAvvv1/09 using the eastern (RSA) primer set.

a sample taken from a sheep just after vaccination with BTV-9 (ITL2003/01), which appears to be identical. In both cases dilution of the RNA test sample resulted in clear detection of the viral RNA, implying that mismatches in the probe site can decrease the robustness of probe binding and its subsequent hydrolysis, to the extent that the assay becomes more susceptible to RT-PCR inhibitors present in the test sample. Variation in the concentration of such inhibitors in the different preparations of RNA may explain why the other sample of the BTV-9 vaccine (RSAvvv1/09) gave consistently +ve results with the combined assay (despite having an identical Seg-1 target sequence), and could therefore have a significant impact on the overall sensitivity and reliability of the assay. The nature and significance of these inhibitors will need further evaluation. The sensitivity of the assay to such inhibitors could potentially be reduced by introducing redundant bases at specific positions in the probe.

The ability of different BTV strains to reassort genome segments in the field has previously been demonstrated (Monaco et al., 2006; Oberst et al., 1987; Sugiyama et al., 1981). The exchange of genome segments could potentially invalidate any diagnostic assays that can only detect one BTV genotype or isolates from a single geographic group, even if used to detect strains of a single serotype (a characteristic that is primarily controlled by genome segment 2 alone). The target footprints for a BTV specific RT-PCR assay, should therefore be as highly conserved as possible across the whole BTV virus species, to ensure that they can be used to detect any BTV strain, without cross-reacting with closely related viruses (like EHDV or AHSV).

BTV Seg-1 (the viral polymerase gene) is one of the most conserved regions of the virus genome (Huismans and Cloete, 1987; Mertens et al., 1987), but has not previously been selected as a target for a BTV specific diagnostic RT-PCR assay. The high level of sequence conservation in genome segment 1 could potentially cause cross-reactions with closely related orbiviruses, as previously reported for Seg-3 (McColl and Gould, 1991). Indeed it was possible to design Seg-1 specific primers that can be used to amplify Seg-1 from both EHDV and BTV (as used for sequence analyses). However, agarose gel electrophoresis showed that relevant cDNA bands were only generated in the rRT-PCR assay described here, when it contained BTV RNA templates (data not shown), indicating that amplification did not occur with the other closely related orbiviruses tested.

In situations where real-time RT-PCR machines are unavailable, the Seg-1 primer sets could be used in a conventional agarose gel based RT-PCR assay to detect BTV RNA. Although the amplicons are small, the conserved nature of Seg-1 suggests that by designing other primers, larger amplicons could be generated, which would be more suitable for electrophoretic analysis, whilst retaining specificity for the BTV serogroup. Since EHDV is recognised as the *Orbivirus* species most closely related to BTV (Mertens et al., 2005), its failure to amplify suggests that the assay will be specific for members of the BTV species.

This study demonstrates the suitability of Seg-1 as a diagnostic target for the sensitive detection of BTV isolates representing any of the 24 serotypes, as well as different topotypes and strains. The sequences of Seg-1 from different BTV strains can be divided into two distinct groups, representing geographically distinct clusters: an eastern genotype (including isolates from the Middle-east, Asia and Australasia) and a western genotype (including isolates from Africa and the Americas). The differences in the Seg-1 sequence made it possible to distinguish between viruses of eastern or western origin by real-time RT-PCR (Fig. 3). Indeed, these assays provided the first indication that the outbreak of BT in northern Europe during August 2006 was caused by a western virus (data not shown). The potential problem of developing a single assay to detect Seg-1 from any of the viruses in these two distinct groups was overcome by combining the two sets of genotype specific primers.

Viral RNA was detected successfully using the combined pan-BTV specific assay with homogenates of *C. sonorensis*, 7–10 days after infection by membrane-feeding with BTV-9 infected blood (Table 2). Individuals or pools of field caught midges could therefore be tested for the presence of virus using this assay, potentially providing information on virus transmission during an outbreak.

The combined assay could be used to detect less than ten template copies of Seg-1 +ve sense ssRNA from the BTV-16 vaccine strain (RSAvvv/16) per reaction. From previous analyses of purified BTV particle infectivity in cell culture, this is equivalent to approximately 0.5 TCID₅₀/ML for disaggregated virus particles in BHK-21 cells (Mertens et al., 1996). This indicates that the assay is approaching the theoretical sensitivity limits of such a system and is significantly more sensitive than the simple detection of virus by isolation in cell culture.

Heat denaturation of viral dsRNA in test samples brings pipetting volumes within the range used by robotic systems, allowing for greater automation of the assay. Automated set-up, when combined with automated nucleic acid extraction, could increase throughput and reduce the steps where there is potential for human error. Heat denaturation also removes the need for a dangerous reagent (methyl mercuric hydroxide) from the RNA denaturation protocol.

Multiple assays with uninfected blood and insect samples, containing ovine, bovine or *Culicoides* nucleic acids, consistently failed to amplify, suggesting that cross-reactions with host sequences would not generate false positive results. The use of the assay to test 2255 EDTA treated blood samples from northern European animals (to confirm disease free status prior to their import to the UK), gave consistently (100%) negative results, indicating that the assay generates only a very low level of false +ve results. Although testing of the combined rRT-PCR assay system on authentic field samples is still in its early stages, positive results were obtained with three out of six samples of sheep blood originally sent to the CRL from the Netherlands at the start of the European outbreak of BTV-8. Two of these samples were also confirmed as BTV +ve by cELISA to detect BTV specific antibodies (Anderson, 1984) and all three were +ve by a conventional Seg-7 specific RT-PCR (Anthony et al., 2007). The remaining three samples were negative in all three assay systems. They were also tested in the Netherlands and it was concluded that these samples were from uninfected animals. Samples of cattle blood taken during the BTV outbreaks in Spain during 2005 were also tested with the combined rRT-PCR assay, giving clear positive results with each of six blood samples, although attempts to repeat virus isolation from one of these samples failed at the CRL. A similar scenario was experienced with three Algerian blood samples which were virus isolation negative but rRT-PCR positive. These results suggest that although the virus in this blood sample was no longer infectious and might therefore be partially degraded, the rRT-PCR assay was still able to confirm its presence and therefore that the animal had been infected.

Clinical samples from remote areas sometimes arrive at the reference laboratory in far from ideal condition, resulting in partial or complete degradation of virus particles and a subsequent loss of infectivity, making virus isolation difficult or impossible. However, the combined Seg-1 assay can be used to detect viral RNA in blood or tissue samples, even where the virus itself is degraded or non-infectious. Indeed viral RNA was detected successfully in sonicated blood samples that had been stored for over a year at +4 °C (data not shown). The assay could also be used to detect non-tissue-culture adapted viruses, and non-infectious virus in the post-viraemic stage of infection (MacLachlan et al., 1994) and it is certainly more rapid than virus isolation.

One unexpected outcome of the study is the development of separate assays (containing only a single primer set) that could be used to detect and distinguish Seg-1 from eastern or western strains of BTV. When combined in a single assay these primers could be used to detect any of the BTV isolates tested regardless of topotype.

The combined assay represents a major improvement in the standard diagnostic techniques used by the CRL to detect BTV. It now forms part of routine testing procedures for samples of blood, tissue, virus isolates and insects by the CRL and has proved to be rapid, sensitive and BTV-specific. Further evaluations and comparisons of this and other real-time RT-PCR assays for the detection of a wide range of BTV strains in field samples, will form part of a future 'ring trial' to be organised by the CRL.

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