Temperature and strain-related variation in the infection and dissemination of bluetongue virus in *Culicoides* (Diptera: Ceratopogonidae)

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Declaration

I declare that all the work submitted in this thesis has been carried out by myself, any collaborative work in acknowledged in the text

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Eva Veronesi

2012

In gratefulness

To all my ghosts

I Abstract

Interactions between bluetongue virus (BTV) and its *Culicoides* vectors in relation to infection, replication and dissemination play a crucial role in the epidemiology of BT. In this study, temperature and strain related factors influencing replication and dissemination of BTV are investigated with a view to providing standardised tools for assessing vector competence. Initially, a Tissue Lyser based assay was developed and optimised to enable quantification of BTV infectious particles and viral RNA in *Culicoides*. This system was paired with a validated real-time RT-PCR assay and used to demarcate transmissible and sub-transmissible infections in orally-infected *Culicoides*. In comparison to virus isolation from cell culture, this technique offered considerable advantages in terms of sensitivity, specificity, repeatability, throughput and robustness. An additional approach to assessing virus dissemination by confocal observation of immunolabelled sections of infected *C. sonorensis* was also successfully trialled.

Quantification of infectious virus on a KC: *C. sonorensis* cell line highlighted differences in replication threshold temperatures across BTV strains. Certain stains were found to replicate at a temperature of 10-12°C, a lower threshold than previously defined for BTV. The use of this assay provides a rapid alternative to replication in *C. sonorensis* females and overcame the issue of high mortality of vectors at low temperatures. Investigation of genotype changes and related infectivity in BTV strains following passage through insect or mammalian cells highlighted a small proportion of amino acid changes in the virus although the impact of these changes during previous studies remains unclear. In addition, the use of *Drosophila melanogaster* as a model for investigation of BTV replication was trialled for the first time and was demonstrated to be a promising model for study of infection and dissemination. The work presented

contributes standardized techniques to investigate interactions between BTV and its *Culicoides* vectors. Key among these are assays to confirm virus dissemination and replication of BTV on cell lines and in vectors under constant temperature regiemes.

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A	Ampere
aa	amino acid
Ab(s)	antibodies
AGE	agarose gel electrophoresis
AHS	African horse sickness
AHSV	*African horse sickness virus*
a.s.l.	above sea level
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
BT	Bluetongue
BTV	*bluetongue virus*
C.	*Culicoides*
cDNA	complementary DNA
CLP	core like particles
CO_2	Carbon dioxide
CPE	cytopathic effect
CSU	Central Service Unit
CRL	Community Reference Laboratory
C_t	Cycle thresholde
°C	degree Celsius
DAPI	4',6-diamidino-2-phenylindole
DB	dissemination barrier
dH_2O	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dsRNA	double-stranded ribonucleic acid
d.p.i.	days post infection
e	eastern
EDTA	ethylene diamine tetra acetate
ECE	embryonated chicken eggs
EIP	extrinsic incubation period
ELD	egg lethal dose
ELISA	enzyme linked immunosorbent assay
agELISA	antigen ELISA
FBS	foetal bovine serum
g	gravity
g	grams
GMEM	Glasgow's modified eagles media
h	hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric Acid
HRP	horseradish peroxidase
IAH	Institute for Animal Health
ICTV	International Committee for the Taxonomy of Viruses
ISVP	infectious subviral particle
IT	intra-thoracic
IU	international units

kbp	kilobase pairs
KC cells	*Culicoides sonorensis (variipenis)* insect cell culture
kDa	kilodalton
Log 10	logarithm to the base 10
M	molar
mAb	monoclonal antibody
MEB	Mesenteron escape barrier
MIB	Mesenteron infection barrier
µg / µl/ µm	micro-grams / micro-litres / micro-metres
Min	minutes
mg / ml	milligrams / millilitres
mM	millimolar
mRNA	messenger ribonucleic acid
n	nano
ns	non structural
nt	nucleotitde
NS2	non-structural protein NS2
NVRL	non-vesicular reference laboratory
O.C.T. compound	optimal cutting temperature compound
OD	optical density
OIE	*Office International des Epizootics*
o/n	over night
OPD	o-phenylenediamine dihydrochloride
OVI	Onderstepoort Veterinary Research Institute
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
rtRT-PCR	real-time reverse transcription polymerase chain reaction
S-1, S-2, (…) ,S-10	segment 1, segment 2, (…) , segment 10
sec	second
SD	standard deviation
SEM	standard error of mean
ss	single stranded
TAE	tris-acteate-EDTA
TCID	Tissue Culture Infection Dose
TPB	Tryptose phosphate broth
TWEEN	Polyoxyethylenesorbitan monolaurate
U	units
UV	ultraviolet
V	Voltage
v/v	volume/volume
VLP	virus like particles
VIB	virus inclusion body
VP	viral protein
w	western
w/v	weight/volume
w/w	weight/weight

Chapter 1: Introduction

1.1 Bluetongue virus

Bluetongue (BT) is an infectious, haemorrhagic, non-contagious arthropod-borne viral disease of domesticated and wild ruminants that was first described in South Africa at the beginning of the 20th century (Spreull, 1905). Bluetongue virus (BTV), the aetiological agent of BT, is a ten-segmented, double-stranded RNA virus belonging to the genus *Orbivirus*, family *Reoviridae* (Mertens *et al.*, 2000; Mertens *et al.*, 2005). BTV is biologically transmitted between its ruminant hosts by adult female *Culicoides* biting midges (Diptera: Ceratopogonidae) and hence requires a period of multiplication and dissemination within the vector (the extrinsic incubation period: EIP) (Mellor, 1990). In addition to suitable environmental conditions, the geographic range and seasonal incidence of BTV is therefore, in part, determined by the availability of competent vector *Culicoides*, in sufficient numbers to sustain transmission (Mellor *et al.*, 2000).

1.1.1 Bluetongue virus: classification, structure and identification

Bluetongue virus is the 'type' species of the genus *Orbivirus* within the family *Reoviridae*, one of eight distinct dsRNA virus families that have been recognized (Mertens *et al.*, 2004a). Reoviruses possess genomes composed of 9, 10, 11 or 12 segments, packaged as a single copy of each linear genome segment within each virus particle; a non-enveloped virus-capsid with icosahedral symmetry; diameters of approximately 70 to 85 nm and a protein capsid composed of up to 3 layers of proteins

(Grimes *et al.*, 1998; Mertens *et al.*, 1984; Mertens *et al.*, 2004b). Members of the genus *Orbivirus* are characterized by their ten-segmented dsRNA genome, packaged within the three-layered icosahedral protein capsid (~85 nm diameter). The inner 'core' of the BTV virus-particle has surface ring-shaped capsomers that can be visualised by conventional electron microscopy and negative staining, hence the name of this genus *Orbivirus* (from the Latin *orbi* = ring, circle) (Borden *et al.*, 1971; Mertens *et al.*, 2004b).

Seven of the ten linear genome segments of BTV encode seven structural proteins (VP1-VP7) and one of the non-structural proteins (NS4) while the remaining three segments encode three further non-structural proteins (NS1, NS2, NS3/NS3a) (Attoui *et al.*, 2009; Belhouchet *et al.*, 2011; Mertens & Diprose, 2004). The genome segments are packaged as a single copy of each per virion, enclosed within the 3-layered icosahedral protein-capsid.

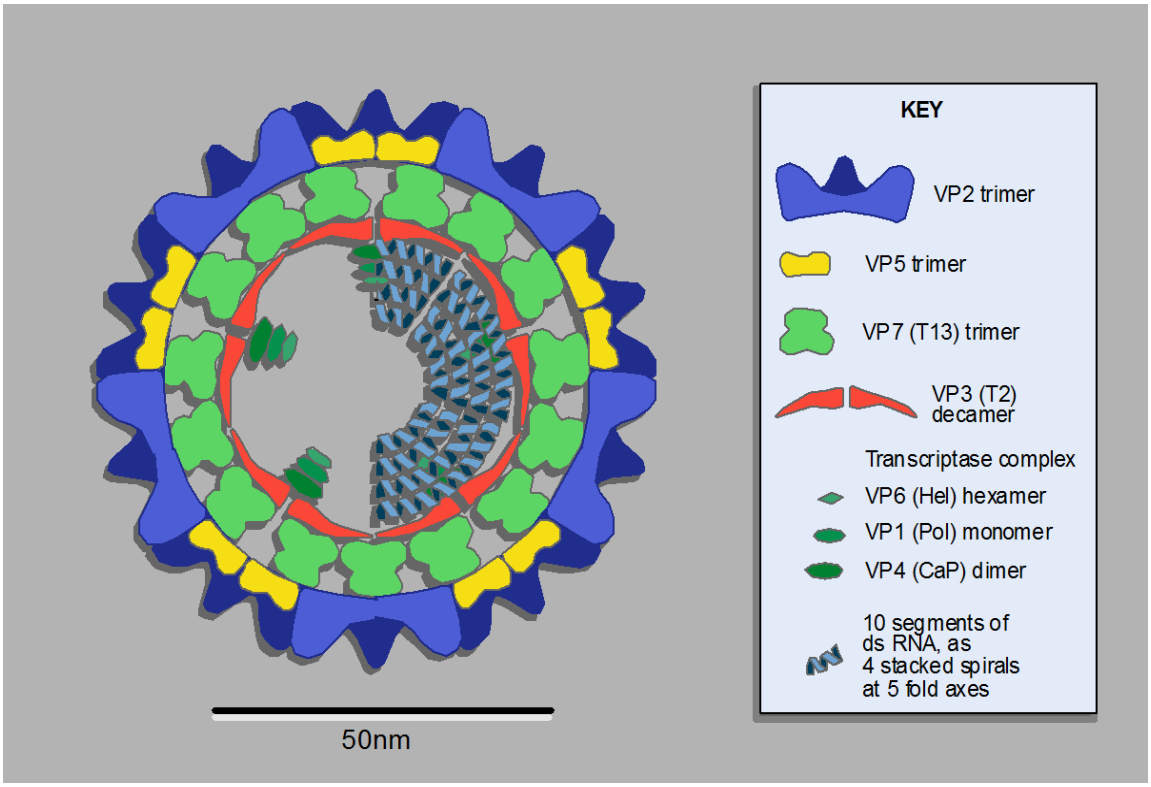

Figure 1.1: Diagram of the bluetongue virus structure, indicating protein location (*reproduced by courtesy of Prof Peter Mertens*)

The BTV capsid is composed of an 'outer capsid' layer, an 'outer core' or 'core-surface' layer and an inner or 'sub-core' shell (Grimes *et al.*, 1998; Huismans & Erasmus, 1981; Mertens *et al.*, 2004a). Genome segments are numbered from one to ten in order of their decreasing size (from 3.9 kb - segment 1 (Seg-1): to 0.8 kb- segment 10 (Seg-10)) (Huismans, 1979; Mertens *et al.*, 2005; Mertens *et al.*, 2004a; Pedley *et al.*, 1988; Wu *et al.*, 1992). The outer capsid layer of BTV is composed of two major virus proteins (VP's) (Verwoerd *et al.*, 1972): 180 copies of VP2 (encoded by Seg-2) and 380 copies of VP5 (encoded by Seg-6) (Burroughs *et al.*, 1995). Phylogenetic comparisons of genome segment two (Seg-2) / VP2 from reference strains of the 26 BTV serotypes, as well as from over 200 other BTV isolates taken worldwide, show a correlation between sequence variation in Seg-2/VP2 and BTV serotype. Within each serotype <33% nucleotide sequence variation has been detected in Seg-2, with 29-59% variation between types (Hofmann *et al.*, 2008; Maan, 2004; Maan *et al.*, 2011; Maan *et al.*, 2007a; Mertens *et al.*, 2007). This relatively high level of variation in segment 2, within an individual serotype, reflects the wide geographic distribution of BTV and the existence of geographic variants or topotypes within each serotype. This allows western (Africa, North or South America) and eastern (Indonesia, India, China or Australia) lineages or topotypes to be distinguished through sequence analysis (Maan *et al.*, 2009).

The outer core layer of BTV is formed by 780 copies of the VP7 protein. VP7 is more highly conserved than either of the two outer capsid proteins and is highly immuno-dominant (Huismans & Erasmus, 1981). Consequently it is the main virus-species / serogroup specific antigen for the different BTV serotypes. However, VP7 does show significant variations both between and within the different topotypes of

BTV (Maan *et al.*, 2008). Consequently, several of the more highly conserved genome segments e.g. Seg-1 or Seg-3, which encode the viral polymerase (VP1), or sub-core shell protein (VP3) respectively have also been used as targets for BTV identification by real-time RT-PCR (e.g. (Shaw *et al.*, 2007)).

1.1.2 Bluetongue virus: replication in cell lines

Following the landmark discovery that BTV could be isolated from ruminant hosts and maintained in embryonated hens eggs (ECE) (Alexander, 1947; Foster *et al.*, 1968), the use of cell cultures for maintenance and detection of BTV became a major component of research programmes worldwide, providing a convenient and consistent framework for a large range of studies. While initially primary bovine lines were investigated, such as calf pulmonary artery endothelial cell lines (CPAE) (Wechsler & Luedke, 1991; Wechsler & McHolland, 1988), there was an increasing tendency to include standard mammalian lines that are not directly epidemiologically relevant to BTV transmission, including baby hamster kidney-21 (BHK-21) and African green monkey kidney (VERO) cell lines (Bando, 1975). Of fourteen different cell lines tested for susceptibility to BTV infection using both cell culture adapted and animal source BTV the CPAE line demonstrated high sensitivity and slightly higher maximum titre than that recorded in BHK-21 cell line (Wechsler & McHolland, 1988). CPAE cells were the only cell line able to result in the isolation of BTV directly from samples, with no prior adaptation of the virus strain, with a frequency similar to that observed in ECE's. However, due to logistic difficulties associated with slow growth rates, the use of CPAE cells has been limited, worldwide.

In addition to mammalian lines, cells derived from insects have also more recently been used in study, maintenance and detection of BTV. The development of

cell lines from embryo's of colonies of the USA BTV vector, *Culicoides sonorensis* was a key advance and the ABADRL-Cv-KC line developed by the Arthropod-borne Animal Diseases Research Laboratory at Laramie has made an important contribution to BTV research since its development in the late 1980 (Wechsler *et al.*, 1989).

Studies of the infection of mammalian and insect cell lines by BTV have revealed detailed mechanisms of both cell entry (Eaton *et al.*, 1990; Hassan & Roy, 1999; Huismans *et al.*, 1983) and exit (Beaton *et al.*, 2002; Eaton *et al.*, 1990; Hyatt *et al.*, 1989; Hyatt *et al.*, 1993; Owens *et al.*, 2004). Although it is still not clear which receptors are involved during the initial binding of BTV virions to the cell-surface membrane, it is thought that this mechanism is mediated by the VP2 protein (Hassan & Roy, 1999; Huismans *et al.*, 1983). The sites where the BTV particles bind are characterised by the presence of clathrin protein (Eaton *et al.*, 1990). The invagination of the virus particle at these sites is followed by acquisition of a clathrin-membrane that eventually detaches from the cell surface-membrane forming vesicles. These vesicles rapidly lose the clathrin-coat and fuse with endosomes present in the cell-cytoplasm.

It has been suggested that the penetration of BTV particles through the cell-membrane could follow a number of different strategies: 1) via receptor-mediated endocytosis (Eaton *et al.*, 1990; Forzan *et al.*, 2007); 2) by direct plasma membrane penetration (Eaton *et al.*, 1990); 3) via an endosomal pathway, possibly mediated by VP7 using glycosaminoglycans interactions and maybe other receptors (Hutchinson, 1999; Xu *et al.*, 1997). Recently (Gold *et al.*, 2010) have described another entry mechanism for BTV-1 which is independent of clathrin and cholesterol but it requires dynamin, delivering virus directly to late endosomes/lysosomes without passing through the early endosome. This pathway shares certain characteristics with macropinocytosis (Swanson & Watts, 1995).

The removal of VP2 and VP5 from the virions to release and activate the BTV cores is low pH dependent (with almost complete release at pH. 6.5 to 6.0) and usually takes place within endosomes (Eaton *et al.*, 1990; Huismans *et al.*, 1983; Hyatt *et al.*, 1993). Cleavage of outer capsid protein VP2, by chymotrypsin or typsin, results in formation of infectious subviral particles (ISVP), which do not possess haemagglutination and aggregation activities (Marchi *et al.*, 1995; Mertens *et al.*, 1987; Mertens & Diprose, 2004). Despite this, ISVP are still infectious with a similar specific infectivity to that of disaggregated virus particles for mammalian cells (BHK-21 cells) (Mertens *et al.*, 1996). ISVP also show a significantly enhanced specific-infectivity to that of the intact virus (approximately × 100) for two insect cell lines (the ABADRL-Cv-KC line and a C6/36 *Aedes albopictus* line) and for adult *Culicoides* (Mertens *et al.*, 1996).

Both VP2 and VP5 of BTV are involved in virus-attachment and penetration of host cell-membranes (Hassan & Roy, 1999). The smaller outer coat protein VP5 can mediate syncytium formation, and is therefore thought to play a role in membrane fusion and penetration that may release the virus core from the endosome into the host cytoplasm (Hassan *et al.*, 2001). Lowering pH (below pH 6.5) releases the outer capsid components from the surface of the BTV core-particle (Eaton *et al.*, 1990; Huismans *et al.*, 1987) and causes structural modification of VP5 (Forzan *et al.*, 2007; Hutchinson, 1999) that may facilitate release of the core from the endosome. Exposure of the virus core by the removal of VP2 and VP5, also allows the core surface protein VP7 to attach to the cell membrane of insect or mammalian cells (Hutchinson, 1999; Mertens *et al.*, 1996). Consequently, BTV core particles are highly infectious to certain insect cells, and adult *Culicoides*. Although the specific-infectivity of BTV-cores for KC cells is similar to that of the intact BTV particles, they are very markedly less infectious for

mammalian cell lines, such as BHK cells, and do not infect CHO cells (Chinese hamster ovary cells) (Hutchinson, 1999). This suggests that the different BTV virus particles enter cells via different mechanisms or routes, which may involve different receptors. ISVPs are the most infectious BTV particle for both KC cells and for adult *Culicoides*, suggesting that they represent the primary route of infection in the vector insect (Mertens *et al.*, 1996).

To avoid antiviral responses, BTV provides its own transcription (VP1) and capping enzymes (VP4) within the virus core. On release of the core from the outer capsid proteins into the host-cell cytoplasm, these enzymes start to synthesise the viral mRNAs necessary for the synthesis of viral proteins and replication, without exposing the dsRNA genome to the host-cell cytoplasm (Mertens *et al.*, 1984; Mertens & Diprose, 2004). The virus synthesises capped mRNA internally within the enclosed core particle, releasing only ssRNA into the cytoplasm of the infected cell (Diprose *et al.*, 2002). Capped mRNAs are translated by the host-cell ribosomes within the infected cell cytoplasm, producing viral proteins and resulting in the formation of granular matrices, termed viral inclusion bodies (VIB).

Assembly of progeny virus particles takes place within these VIB. Although the exact mechanism of virus genome assembly and replication is still not clear, the VIB have been shown to contain large amounts of viral proteins (including VP1, VP3 and VP7 as well as the ssRNA and dsRNA-binding proteins VP6 and NS2) (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/BTV.htm) (Hyatt *et al.*, 1992; Roy *et al.*, 1990a). Sub-cores, which have a single capsid layer composed of VP3 and are considered unlikely to be infectious, are formed within the VIB. Although these can be purified for some *Orbivirus* species (e.g. equine encephalosis virus) they are relatively unstable, suggesting that they are a transition state during assembly or breakdown of the

intact virion (Peter Mertens, personal communication). The BTV core surface layer also appears to be assembled within the VIB matrix (Brookes *et al.*, 1993; Eaton *et al.*, 1990; Mertens & Diprose, 2004). The presence of VP2 and VP5 at the margin of the VIB indicates that they are added to form the outer capsid layer as the particles exit from the VIB and enter into the cytoplasm.

The mechanism by which BTV is released from the host cell is less clear but may involve up to four different mechanisms. Firstly, the BTV particle may exit via budding from the surface of infected mammalian cells, acquiring a membranous envelope from the cell surface membrane in the process (Fu, 1995; Hyatt *et al.*, 1989). These particles are infectious but the membrane is thought to be unstable, and the role they play in progression of the disease in infected animals is still unknown (Peter Mertens, personal communication). Secondly, the small non-structural proteins NS3 and NS3a can mediate release of virus particles from infected insect cells (Hyatt *et al.*, 1991; Hyatt *et al.*, 1993; Roy *et al.*, 1990b). Thirdly, there is also evidence from electron microscope-based studies for release of BTV particles by direct penetration of the cell membrane (Fu, 1995), which also results in gaps in the cell membrane and release of some cellular components, a process that may lead to cell lysis. Finally, BTV infection leads to severe cytopathic effects (CPE) in most mammalian cells, culminating in cell lysis and the release of progeny virus particles from the cell cytoplasm.

While a fundamental tool for BTV research, adaptation of the virus through repeated cell passage can generate or select specific and possibly non-representative sub-populations from the quasi-species, resulting in changes of their overall biological phenotype (Weaver, 2006; Weaver *et al.*, 1999). Until relatively recently, the vast majority of field derived BTV strains were isolated through passage of blood samples from infected ruminant hosts in either mouse brains or ECE's. These were then usually

passaged blind on mammalian cell lines (most commonly BHK-21 cells) until a cytopathic effect (CPE) became observable following application of the sample to cells. Increasingly, however, the ABADRL-Cv-KC *C. sonorensis* cell line is being used for the purpose of isolation, as other diagnostic assays are now more commonly available that do not require the observation of CPE. While still selective, it is assumed that isolation and virus growth in KC cells, represents a more realistic population bottleneck than standard mammalian lines, and requires fewer repeated passages of BTV's for use in laboratory studies.

1.1.3 Bluetongue virus: History and Current Status in Europe

BT was first described following importation of naïve susceptible Merino sheep from Europe into South Africa and was initially described as 'Malarial Catarrhal fever' (Hutcheon, 1902). Later, the disease was re-named 'bluetongue' due to the cyanotic appearance of the tongue in certain individuals exhibiting clinical signs (Spreull, 1905). In susceptible animals, BT is characterised by pyrexia, lameness, depression, oedema, and in serious cases respiratory distress and death. This characterisation was closely followed by the discovery that BTV was an agent that could be filterable and that it could be transmitted to cattle and goats, but that these hosts did not suffer apparent clinical disease (Spreull, 1905). From the 1940's until the 1980's, BTV was viewed as an emerging virus and strict restrictions on the movement of ruminants and derived products (e.g. semen) were employed to reduce the probability of its spread (Schudel *et al.*, 2004). The OIE also registered BT as a 'List A' notifiable disease, a status reserved for major pathogens of livestock (OIE, 2007). Increasingly, however, evidence arose that BTV had been circulating in many regions with only transient disease incidence (MacLachlan, 2010). This almost silent circulation of BTV occurred through a combination of resistance to clinical disease in local sheep breeds and the fact that BTV

incidence was hugely underestimated in regions dominated by susceptible livestock species other than sheep (e.g. cattle) that did not usually show clinical signs. The length of time for which BTV had been circulating in many of these regions remains poorly characterised.

Evidence of clinical BT in Europe (in Cyprus) was recorded anecdotally in the 1920s, but the disease was only officially recognised outside of the African continent in the early 1940s when a severe outbreak occurred in Cyprus and Palestine (Gambles, 1949). Sporadic outbreaks of the virus subsequently occurred in Portugal and Spain in 1956, with a loss of about 180,000 sheep (Lopez & Sanchez, 1958a; Lopez & Sanchez, 1958b; Manso Ribeiro *et al.*, 1957; Mellor *et al.*, 1990; Mellor *et al.*, 1983) and in 1979 the disease was found in the Greek islands of Lesbos (Vassalos, 1980) and Rhodes (Dragonas, 1981). Following these outbreaks, BTV did not spread further and in Greece the restriction and control measures employed (based upon destruction of infected individuals), succeeded in eradicating the virus (Nomikou *et al.*, 2004).

During the 1990's it was increasingly suggested that BTV circulation within certain geographic regions was restricted by barriers to virus movement that might be used to facilitate safe trade movements between BTV endemic and free areas. The validity of this view rested largely on serological data which appeared to show that the variety of serotypes of BTV found in specific countries was remarkably stable (e.g. Australia: (Kirkland, 2004; Pritchard *et al.*, 2004), USA: (Heidner *et al.*, 1991; Kowalik & Li, 1987)). In addition, limited data were also presented to suggest that major indigenous vector species in one of these so-called 'episystems', North America (Gibbs & Greiner, 1994; Tabachnick, 2004), had a reduced competence for exotic strains of BTV. OIE list includes viruses that have the potential to cause large scale transboundary diseases outbreaks. The 'episystem' theory hypothesis

therefore suggests that exotic BTV strains might not be able to colonize new regions effectively and therefore, that BTV might be removed from the OIE's List A as the designation of this status had originally been driven by the view that BTV was an emerging disease.

1.1.3.1 BTV in Europe 1998-2006.

In 1998 BTV was discovered in Anatolian Turkey, the first of multiple incursions of the virus into the Mediterranean basin that occurred in what was widely regarded at that time as the economically most damaging outbreak of BT in recorded history (Baylis & Mellor, 2001; Calistri *et al.*, 2003; Mellor, 2004b; Mellor & Wittmann, 2002; Purse *et al.*, 2005; Zientara *et al.*, 2000). Unlike previous outbreaks of BTV in this region, analyses and comparisons of the full-length nucleotide sequences of BTV Seg-2 for the first time provided a clearer understanding of individual virus lineages, relationship and origins (Maan *et al.*, 2004a; Maan *et al.*, 2004b; Mertens *et al.*, 2007). A total of seven separate BTV strains from six serotypes (BTV-1, 2, 4, 9 and 16) were identified that had entered Europe using four distinct routes through Asia (eastern topotypes) and Africa (western topotypes).

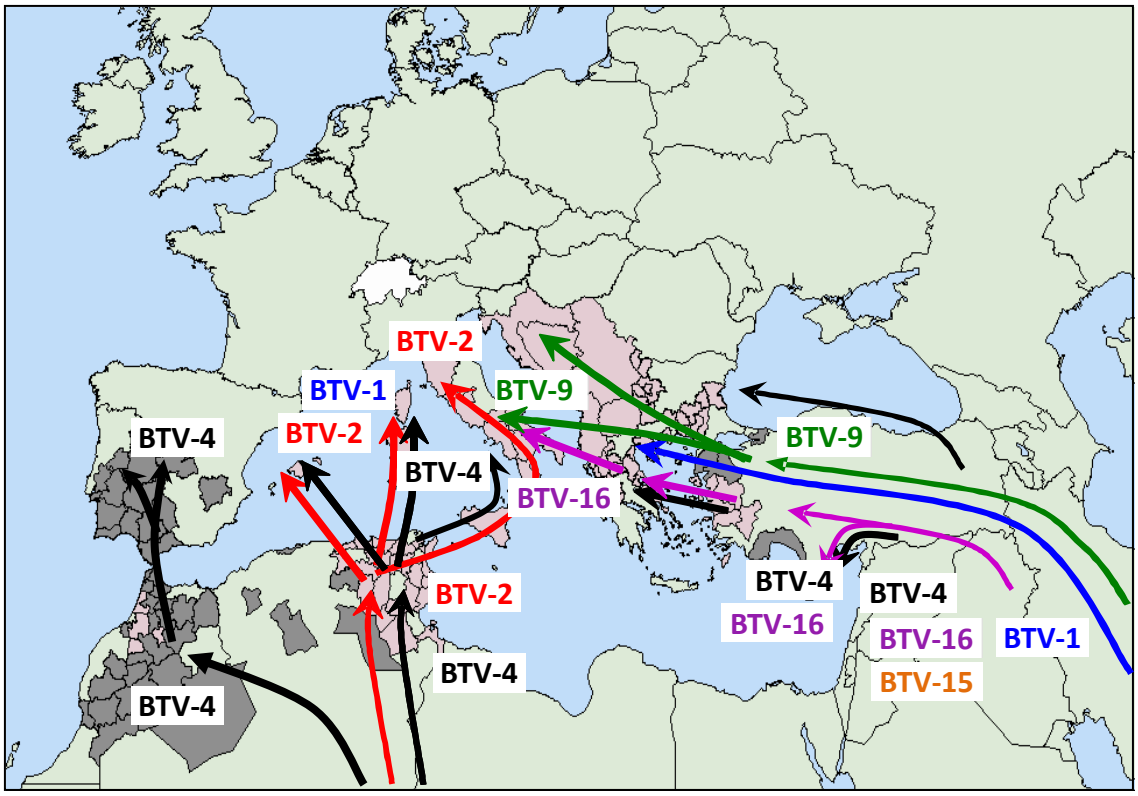

Figure 1.2: Introduction of different strains of BTV in Europe during 1998-2006
(Reproduced by courtesy of Prof Peter Mertens)

The use of novel sequencing technologies was key in enabling strains of the same serotype to be phylogenetically compared for the first time and identified two separate topotypes of BTV-1 circulating in Europe. An eastern topotype of BTV-1 (with close similarity to those from India and Malaysia), was isolated in Greece in 2001 and was hypothesised to have entered Europe via Turkey (Maan *et al.*, 2009; Nomikou *et al.*, 2009). Additionally, a western BTV-1 topotype was identified in 2006 which shared a high similarity to strains from sub-Saharan Africa (95.9% nucleotide identity: (Maan *et al.*, 2009)). This strain is thought to have entered Europe from North Africa (Algeria, Libya and Morocco in 2006) (Cetre-Sossah *et al.*, 2011) spread to Sardinia in 2006 (Cetre-Sossah *et al.*, 2011) and Portugal and Spain in 2007 (Calvete *et al.*, 2008; Fernandez-Pacheco *et al.*, 2008).

A second example of the power of these techniques lay in the identification of strain origin, in particular in differentiating incursions of field strains and the emergence of live-attenuated vaccine strains of BTV, as a major source of damaging outbreaks of BT. This is illustrated by the emergence of BTV in Greece (Vassalos, 1980). The first outbreak of BTV in Greece in 1998 was originally thought to be caused by BTV-4, on the basis of previous incursion of this serotype and the use of a live attenuated vaccine (LAV) against it in Turkey (Commission, 2000). Phylogenetic analysis of this strain, however, demonstrated it to be BTV-9 (Anonymous, 2000) and hence unrelated to the Turkish Vaccination Programme. The Turkish BTV outbreak was also later confirmed to be caused by BTV-9 and not by BTV-4 (Mellor & Wittmann, 2002). Between 1999 and 2000, widespread outbreaks of BTV-4 were also recorded in Greece**.** Phylogenetic analyses demonstrated that this virus was similar to earlier isolates from Cyprus and Turkey and not from the BTV-4 LAV previously used in Turkey (**www.reoviridae.org/dsRNA_virus_proteins/btv4-segment-2-tree.htm**).

Later phylogenetic analyses of segment 2 identified BTV-16, initially isolated in Greece during 1999-2000 (Mellor & Wittmann, 2002), demonstrated close similarity to the strain circulating in Turkey and almost identical sequences (<0.7% nucleotide sequence difference) to the reference and LAV strains originally produced in South Africa (Maan *et al.*, 2009). The entrance route for BTV-16 in Europe was most likely via Turkey, as strains from this area (Greece and Cyprus) form a closely related group (Maan *et al.*, 2009). Moreover, the BTV-16 that appeared in mainland Italy in 2002 (Giovannini *et al.*, 2004) was even more closely related to the Israeli vaccine strain (99.9% nucleotide identity), suggesting it was not derived from the outbreak in Turkey and Greece but may have been derived separately from the live attenuated vaccine used in Israel for several years as part of a multivalent vaccine campaign (Batten *et al.*,

2008b). The Italian strain of BTV-16, however, was later found in Sardinia and was subsequently thought to have been derived from the Italian vaccination campaign conducted with LAV BTV-16 produced in South Africa (Monaco *et al.*, 2006).

1.1.3.2 BTV in Europe 2006-2008

In August 2006 BTV emerged for the first time in recorded history in northern Europe with the discovery of diseased sheep in the Maastrict district of the Netherlands (Diseases, 2007b; Maan *et al.*, 2008; Toussaint *et al.*, 2007b)). This outbreak occurred at a latitude of 53ºN (Mellor *et al.*, 2009), far further north than ever previously recorded in mainland Europe. RT-PCR assays and subsequent sequencing/ phylogenetic analyses showed that the outbreak was caused by a strain BTV-8 which shared in Seg-2 97% nucleotide identity with a western strain originating in sub-Saharan Africa (Maan *et al.*, 2008), although few isolates of BTV-8 were available for comparison (Mellor *et al.*, 2009). The route by which the virus entered Europe is unknown, however this remains the subject of considerable speculation (Mintiens *et al.*, 2008).

During subsequent months BTV-8 spread to neighbouring countries (Belgium, mainland France, Germany and Luxembourg) (Elbers *et al.*, 2008; Wilson & Mellor, 2009b) affecting over 2000 holdings (Losson *et al.*, 2007). New cases of BT finally ceased during late autumn 2006 when falling temperatures prevented completion of the EIP and reduced adult populations of *Culicoides* during the winter of 2006/7. A major point of uncertainty at this time lay in the probability of overwintering of the BTV-8 strain in northern European conditions (Wilson & Mellor, 2008).

Three possible mechanisms of overwintering have been suggested.

Firstly, an overwintering mechanism in the ruminant hosts have been postulated. While initial studies of long-term persistence of BTV in ruminant hosts in

the USA suggested that this could occur (Luedke *et al.*, 1977; Nevill, 1971) these studies were later criticised for not using vector proofed facilities, raising the possibility that re-infection was occurring in the subjects. Later studies suggested that persistently infected gamma-delta (γδ) T-cells could be a persistent source of BTV in infected hosts, however, several other studies had been unsuccessful in replicating the long-term recovery of BTV (Lunt *et al.*, 2006; Melville *et al.*, 2004; White & Mecham, 2004).

Secondly, additional studies had considered transovarial transmission in vectors as a potential means of overwintering in the USA, however, these studies had failed to demonstrate the presence of infectious BTV in offspring under laboratory conditions using a variety of diagnostic techniques (Ballinger *et al.*, 1987; Fu *et al.*, 1999; Jones, 1971; Nunamaker *et al.*, 1990). Hence, while viral RNA in *Culicoides* larvae from endemic BT areas has been detected no studies have demonstrated the presence of infectious BTV in field collected larvae of *Culicoides* (White *et al.*, 2005).

Thirdly, a possible mechanism of BTV-8 overwintering lay in vertical transmission of BTV within the ruminant host via transplacental transmission. This phenomena was initially postulated based on preliminary observation of “dummy lambs” born from pregnant ewes that were previously immunized with live attenuated vaccines for BTV (Schultz & Delay, 1955). The mechanism was then fully investigated in the late 1970s (Gibbs *et al.*, 1979), suggesting different stages of infection in calves according to their gestation period. The authors reported that calves infected before their immune system is fully developed demonstrated a prolonged infection of up to 2 months after birth. A major consideration in this experiment, however, was the use of cell-passage adapted BTV strains to infect the cattle, which renders the application with field collected data less realistic. When combined with data from the field, this led to an

assumption that transplacental transmission was only associated with these strains (MacLachlan *et al.*, 2000).

In April 2007, seroconversion for BTV-8 was recorded in Germany in a sentinel animal, indicated that the virus had successfully overwintered in this region (Diseases, 2007a; Wilson *et al.*, 2007). Later studies demonstrated that the BTV-8 strain circulating in Northern Europe during the 2006 outbreaks could cross the bovine placenta in a high proportion of cases and infect calves when dams are infected during pregnancy (Darpel *et al.*, 2009; De Clercq *et al.*, 2008). Whether this is the primary method of overwintering of BTV in this region remains unknown. Following its re-emergence in 2007, BTV-8 continued to spread and was reported in the United Kingdom during September 2007 (Maan *et al.*, 2008; Wilson & Mellor, 2009a). In 2007 and 2008, the full extent of clinical impact of BTV-8 infection became apparent, including the observation of disease in cattle and cases (with a high percentage of fatalities in sheep) in thousands of holdings across the affected region.

Concurrently, a western lineage of BTV-1 from Morocco (Diseases, 2006a; Diseases, 2006b; c) was spreading northwards, reaching Spain (Anonymous, 2007c) Portugal (Anonymous, 2007b) and eventually the northern part of France, by November 2007 (Anonymous, 2007a) and eventually spreading northward reaching northern coast of France (Brittany). In addition, two further strains of BTV were discovered in 2008 of BTV-6 and BTV-11 serotypes, associated with apparent illegal use of live attenuated vaccines (De Clercq *et al.*, 2009; Eschbaumer *et al.*, 2010). Sequence analysis of Seg-2 revealed 100% identity with the South African BTV-6 live-vaccine-strain (Maan *et al.*, 2010). In addition, two segment seven sequences were detected in the isolated BTV and the original blood sample from the infected ruminant, one of which was almost identical to the BTV-16 vaccine strain (99.7 nucleotide identity), and the other to the Netherlands

BTV-8. This suggested co-infection with both BTV-8 and BTV-6, and that the virus was in the process of re-assortment with the northern field strain of BTV-8. Unlike BTV-8 and BTV-1, neither of these vaccine strains produced noticeable clinical disease in infected ruminants and their appearance was transient, failing to successfully overwinter in the region.

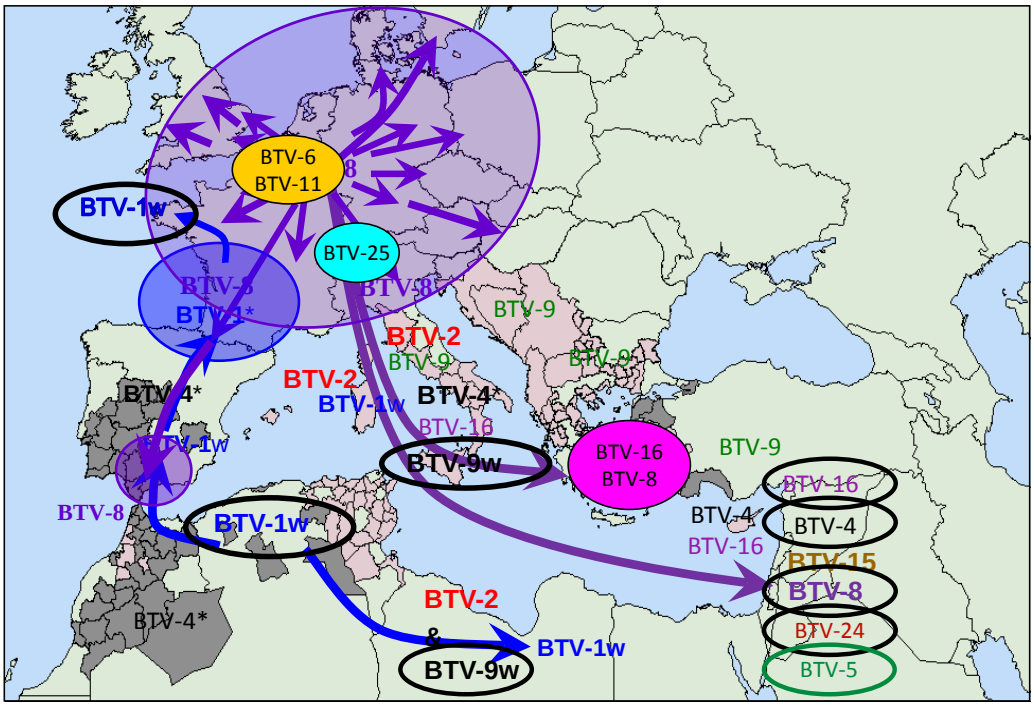

Figure 1.3: Bluetongue virus strains present in Europe during 2006-09
(Reproduce by courtesy of Prof Peter Mertens)

An additional strain of BTV was also discovered in 2008 in a flock of goats in Switzerland (Toggenburg) through antibody ELISA and viral RNA detection (Hofmann *et al.*, 2008) despite a lack of clinical signs. Segment-2 sequencing demonstrated an identity of only 63% with any of the other 24 BTV reference strains (Hofmann *et al.*, 2010) and hence this strain represented a new BTV serotype, BTV-25.

1.1.3.3 BTV in Europe 2009-Present

The unprecedented incursions of multiple BTV strains into Europe since 1998, has led to a general reassessment of methods used to control the virus, both in terms of legislation and practical methods used by farmers to reduce incidence in their stock. Control of animal movements from infected premises (i.e. risk zones), regardless of whether animals were infected or not, was one of the major strategies to reduce disease spread. While an essential strategy in preventing long-distance introduction of BTV strains into new regions, these movement controls are damaging to the livestock industries and are difficult to maintain in the long-term due to their economic impact. Initial controls in the EU also included slaughter of affected livestock (most notably in Greece where BTV was eradicated using a combination of techniques) and insecticidal control of vectors. The scale and intensity of BTV outbreaks from 1998 to 2005, however, demonstrated that this strategy could have serious consequences if not successful in the early stage of an outbreak, leading to the slaughter of some one million affected animals in this region prior to the implementation of vaccination (Purse *et al.*, 2005).

While many approaches have been evaluated for control of *Culicoides*, these have largely centred upon reducing biting nuisance, rather than interrupting virus transmission (Blackwell *et al.*, 2004; Carpenter *et al.*, 2008b; Trigg, 1996). Despite having a long history of use during BTV outbreaks in the EU, the quantitative impact on populations of *Culicoides* or BTV transmission is almost non-existent (Mullens *et al.*, 2001). In southern Europe, general maintenance interventions that individual farmers can apply, such as elimination of standing water pools, leaky irrigation pipes, and manure or substrate reach in organic content would be expected to impact upon breeding sites of *Culicoides*. In northern Europe, however, where larval habitats are less

restricted and multiple species are thought to drive BTV epidemics, the effect of these measures would be limited. In addition, the use of insecticides is now heavily restricted in legislation applied across the region. This led to the common use of pour-on or dip-wash formulations of pyrethroids already in use for control of arthropod-borne diseases (Eisler *et al.*, 2003) with the aim to induce a significant mortality rate in those midges that land on the animal and a reduction on their feeding rate has also been recorded (Habtewold *et al.*, 2004; Mullens, 1993). However, to date, no field trials have confirmed their efficacy in reducing BTV transmission in the field (Mullens *et al.*, 2001).

Prevention of viraemia in BTV susceptible hosts can also be attained through the use of vaccines (Alpar *et al.*, 2009; Noad & Roy, 2009; Roy *et al.*, 2009; Savini *et al.*, 2008). Live attenuated vaccines (LAV) were first produced in South Africa (Dungu *et al.*, 2004) and have been extensively used, particularly in BTV endemic regions, for the control of sporadic outbreaks of BT. BTV LAV were initially also produced in the United States against serotype 10 (Kemeny & Drehle, 1961; Luedke & Jochim, 1968) and are now commercially available also for serotypes 11 and 17. From early in their history, many concerns have arisen surrounding the use of LAV (Commission, 2000). Where insufficiently attenuated for the target host population, they may elicit viraemia in vaccinated animals that is at a sufficient level to allow vector *Culicoides* to become infected and transmit the virus to a new host (Venter *et al.*, 2007). They may also inflict clinical signs in susceptible breeds of sheep, as was the case with the live vaccines used during incursions of BTV in southern Europe (Ferrari *et al.*, 2005; Veronesi *et al.*, 2010; Veronesi *et al.*, 2005). These LAV can also cause teratogenic effects when used in pregnant animals (Flanagan & Johnson, 1995). Finally, reassortment of genome segments between vaccine virus and field strains in dual infected vectors and/or hosts,

could potentially result in viruses with altered virulence characteristics (Batten *et al.*, 2008b; Brenner *et al.*, 2010; Cowley & Gorman, 1989; Monaco *et al.*, 2006; Sugiyama *et al.*, 1981).

In part, driven by an understanding of the limitations and potential problems with of LAV, inactivated BTV vaccines have also been developed for use in epidemic areas and were deployed with great success to eradicate BTV-8 from northern Europe (Eschbaumer *et al.*, 2010) with most affected countries in this region being declared BT free by 2011. When produced correctly, inactivated BTV vaccines should not contain infectious virus and therefore have the advantage of a low probability of transmission by *Culicoides* or reversion to virulence (Veronesi *et al.*, 2010; Veronesi *et al.*, 2005). There are certain drawbacks, however, that include the possibility of incomplete or short lived host protection, due to insufficient development of neutralizing antibodies, and limited cross protection if used in multi-serotype inoculations (Erasmus, 1980). The inactivated vaccines that were used in northern Europe contained relatively crude tissue culture grown virus that was chemically inactivated (Moulin *et al.*, 2012). As a consequence they contain all of the virus structural and non-structural proteins, which are all likely to raise antibodies in the vaccinated animals. In the face of a widespread vaccination campaign this prevents separation of naturally infected from vaccinated stock using the currently available serological assay for diagnostic purposes as this relies on antibody detection. Surveillance of the BTV outbreaks was therefore entirely dependent on nucleic acid based assays (RT-PCR) to detect infected animals. In addition, due to their limited shelf life (Hammoumi *et al.*, 2003) (Ronchi *et al.*, 2003), inactivated BTV vaccines are rarely available in vaccine banks that could be used to eradicate outbreaks in their early stages and while still relatively inexpensive to produce, cost of production remains somewhat higher than LAV's.

There has also been a considerable body of research aiming to produce BTV vaccines that can provide cross-serotype immunity. Possible candidate are “virus-like particles” (VLP) (Roy, 2004) which are formed by the major BTV structural proteins that mimic native virus particles and are therefore thought to raise a more ‘natural’ immune response. ‘Recombinant vaccines’ have also been used to express a limited number of the viral proteins (including VP2), to raise a neutralising and protective response (Boone *et al.*, 2007), or can incorporate markers. This makes it possible to identify vaccinated animals by detection of antibodies to the markers. Subunit vaccines are also compatible with a DIVA (Differentiating Infected from Vaccinated Animals) assay, for example by detection of antibodies to non-structural proteins that are not included in the vaccine, but are expressed during virus replication (Alpar *et al.*, 2009). These recombinant ‘next generation’ vaccines are regarded as safe and do not have a risk of transmission of BTV. They are usually, however, still type-specific and since they are not yet available for all BTV serotypes, they would still need to be developed for the individual serotype(s) causing a specific outbreak(s). Consequently there is a significant cost and time delay associated with their development and application. Further research is also needed concerning the choice of delivery system, of adjuvants or micro-bead carriers (Roy *et al.*, 1990c; Somavarapu *et al.*, 2003). Recent studies show promising results by introduction of the conserved NS1 protein into the composition of sub-unit vaccines against BTV, generating a cross-protective response against unrelated serotypes, via a cross-reactive cellular immune response. (Calvo-Pinilla *et al.*, 2012)

The repeated incursion of multiple different BTV strains into the EU has demonstrated that the epidemiology of this virus has changed. The advent of improved sequencing technologies and creation of better nucleic acid based detection and typing

methods (Caporale *et al.*, 2011; Maan *et al.*, 2012a; Maan *et al.*, 2007b), including relevant virus collections and sequence databases, have improved our surveillance capabilities. These new capabilities have demonstrated that the entire European region is vulnerable to further incursions of BTV, via at least four different routes (Maan *et al.*, 2009; Wilson & Mellor, 2008). Re-assortment must also be considered a major source of potential phenotypic variation in overlapping strains in the field (Batten *et al.*, 2008b; Maan *et al.*, 2012b; Sugiyama *et al.*, 1981). These developments require new tools (new vaccines) to reduce the impact of future outbreaks. Strategies for BTV control, however, require consideration of the relatively low commercial value of the animal (primarily sheep) making the cost of vaccines a major driving factor in the current choice of vaccination technology.

1.2 *Culicoides* biting midges

1.2.1 Ecology and lifecycle

Culicoides (*Latreille*) are among the smallest haematophagous flies with a body size that ranges from one to three millimetres in length (Mellor *et al.*, 2000). They have been recorded in a wide range of habitats, at altitudes (up to 4000m) and have a worldwide distribution with the exception of Antarctica and some island territories (Mellor *et al.*, 2000). Taxonomically, *Culicoides* are placed within the family Ceratopogonidae which includes over 134 genera and 6321 species worldwide (e.g. http://www.inhs.uiuc.edu/research/FLYTREE/Borkent.html).

A total of 1360 species of *Culicoides* has been described to date (Boorman & Hagan, 1996; Borkent & Wirth, 1997; Kettle, 1984). However, an inability to differentiate intra- and inter-specific variation in morphology means that to date a large but as yet undetermined number of species has been overlooked (Meiswinkel, 1989;

Meiswinkel *et al.*, 2004a; Waller *et al.*, 1987). Broad advances in molecular techniques across medical and veterinary entomology have supported development of techniques to differentiate morphological similar species within major vector groups (Linton *et al.*, 2002; Lunt *et al.*, 1996; Mathieu *et al.*, 2007; Sebastiani *et al.*, 2001). Exploiting these advances, internet-based resources have also been developed that track both species numbers and phylogenetic classification (e.g. www.culicoides.net).

Culicoides are holometabolous, passing through four life stages: egg, larvae, pupae and adult (Figure 1.4). The duration of their lifecycle (from egg to adult) can range in length from a few weeks for species in tropical regions, to several years for those in the arctic (Downes, 1962), reviewed by (Borkent, 2005). In general, the egg and pupal stages of development are of short duration, while the larval stage requires the longest time for completion. Larval development encompasses four instars and ranges in duration from 8-10 days in the case of the afro-tropical, dung-breeding *C. bolitinos*, to two years in some arctic species (Downes, 1962; Meiswinkel, 1989). While arrested development (diapause) in temperate climates, has been suggested as both eggs and larvae, the exact mechanism of this phenomenon remains poorly investigated (Jobling, 1953; Kettle, 1965; Mullen, 2002).

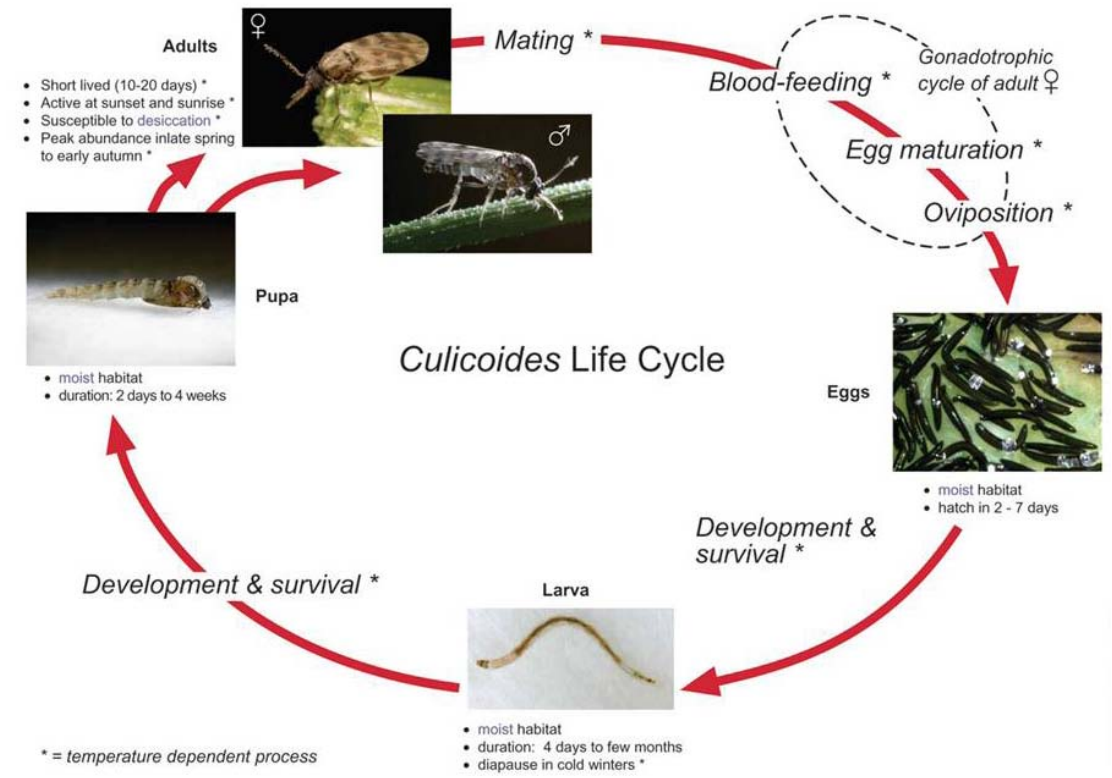

Figure 1.4: *Culicoides* life Cycle (reproduced from *Purse et al. 2005*)

Culicoides larvae develop in a diverse range of habitats, but are highly vulnerable to desiccation and hence tend to be restricted to relatively permanent, semi-aquatic conditions (Blanton & Wirth, 1979; Kettle, 1984; Kettle & Lawson, 1952). In many *Culicoides* species, the restricted nature of their larval development sites and their relatively poor directional flight ability leads to the vast majority of emerging adults adopting only local dispersal (Kettle, 1984). Field experimentation based on the recapture of marked wild caught larvae of *C. sonorensis* has demonstrated a maximum flight range of up to 4 km in small numbers of individuals (Lillie *et al.*, 1981). In rare cases, rapid changes in the distribution of *Culicoides*-borne arboviruses, in the absence of livestock movements, have been used to infer very long distance semi-passive (wind borne) adult flights of hundreds of kilometers (Gloster *et al.*, 2007a; Gloster *et al.*, 2007b; Sellers *et al.*, 1977; 1978; Sellers, 1980).

The adult *Culicoides* life span is rarely estimated in the field but most species are thought to survive for 1-3 weeks (Birley & Boorman, 1982; Mullens & Rutz, 1984; Work *et al.*, 1991), although this may be extended considerably in individuals kept under laboratory conditions (Campbell & Kettle, 1976; Hunt, 1994; Jones, 1964; Koch & Axtell, 1978; Veronesi *et al.*, 2009). Adults of the majority of *Culicoides* species are crepuscular and/or nocturnal, although a few species appear to have lost this habit secondarily (Reuben, 1963). Female *Culicoides* are almost all haematophagous, feeding upon a variety of different host animals and exhibiting varying degrees of host specificity (Bennett & Fallis, 1960; Braverman & Phelps, 1981; Downes, 1958; Hair & Turner, 1968).

1.2.2 Culicoides as arbovirus vectors

Culicoides act as vectors for a range of economically important veterinary arboviruses and more than 50 arboviruses have been identified from them worldwide (Meiswinkel *et al.*, 1994; Mellor, 2000). The most important of these are bluetongue virus (BTV), African horse sickness virus (AHSV), epizootic haemorrhagic disease virus (EHDV), Equine encephalosis virus (EEV), Akabane fever virus (AKAV), bovine ephemeral fever virus (BEFV) and Palyam viruses (Mellor *et al.*, 2000). In addition, *Culicoides* also transmit nematodes and protozoa to both livestock and humans (Linley *et al.*, 1983; Mellor *et al.*, 2000) and can cause a severe allergic dermatitis (commonly called ‘sweet itch’) in horses (Braverman *et al.*, 1983). At present BTV is by far the most economically important *Culicoides*-transmitted pathogen worldwide (Carpenter, 2009; Carpenter *et al.*, 2009; Tabachnick, 1996a).

The transmission of arboviruses to their mammalian hosts during feeding by *Culicoides* is dependent on the initial acquisition of the virus and its subsequent

replication and dissemination in individual females of competent vector species (Foster *et al.*, 1963; Mellor, 1990; Mellor, 2000). Not all *Culicoides* species or individuals are susceptible to BTV infection and even if they can become infected they may still be unable to transmit the virus. Many parameters determine the ability of an arthropod to act as a vector for a specific arbovirus, which are generally grouped within the concept of vectorial capacity. Vectorial capacity refers to the overall probability that a vector-borne pathogen will be transmitted, given a defined range of internal and external parameters (Garrett-Jones & Shidrawi, 1969) . Vector competence is one of these parameters and is commonly defined as the ability of an arthropod or population to acquire, support, and transmit the pathogen (Reisen, 1989a; b).

The vector competence of an arthropod for a specific arbovirus is commonly quantified experimentally in the laboratory by feeding the putative vector on a source of the pathogen, incubating for a given time period (usually under constant temperature) and then assessing pathogen dissemination and hence the potential for onwards transmission (Hardy *et al.*, 1983; Meyer *et al.*, 1986). A key consideration is not only whether the vector can support amplification of the virus, but also whether it can transmit the pathogen to a susceptible host following feeding (Hardy *et al.*, 1983; Mellor, 1990; Mellor *et al.*, 2000). While the early definitions of vector capacity and vector competence were developed for the malaria-mosquito system (Gambles, 1949; Hardy *et al.*, 1983; Kramer *et al.*, 1992; Mitchell, 1983), these concepts have subsequently been applied to many other pathogen-vector relationships and form a basis for models of transmission (e.g. for BTV: (Gubbins *et al.*, 2008; Mullens *et al.*, 2004)).

1.2.2.1 Culicoides *and BTV replication*

The first evidence implicating *Culicoides* in transmission of BTV was produced at the Onderstepoort Veterinary Institute in South Africa during the 1940's (Du Toit, 1944). In this work, naïve, susceptible sheep were shown to develop typical BTV clinical signs and pyrexia following injection with homogenates of *Culicoides* collected using a light-suction trap. In addition, in one of very few animal-based transmission experiments carried out using *Culicoides*, clinical signs and elevated temperature were also recorded in another sheep bitten by *Culicoides* previously fed upon a 'donor' BTV viraemic sheep (Du Toit, 1944). Unfortunately, only a few engorged *Culicoides* were recovered from this experiment, but these were tentatively identified as *Culicoides pallidipennis* (later re-assigned as *Culicoides imicola*).

Techniques used during this initial study were later criticised (Foster *et al.*, 1963), although the issues raised were well understood and in fact discussed by the original author (Du Toit, 1944). Key among these was the fact that sheep were not kept in a vector-proof accommodation in a region of endemic BTV transmission, raising the possibility of contaminating infections. A second major limitation also lay in the fact that, the only detection systems available at the time centred upon observation of clinical cases and pyrexia, and the fact that the hosts used had the potential to have been previously infected, and hence immune, to the viruses used.

Noting these limitations, later studies carried out in the USA attempted to demonstrate the role of *Culicoides* in transmission of BTV in a more convincing fashion. An important development enabling these studies was the production of the first vector-competent colonies of *Culicoides* worldwide in 1957 (Jones, 1957; 1964) and membrane-based techniques for feeding (Rutledge *et al.*, 1964). Somewhat fortuitously, the major vector species identified through preliminary studies in the USA

was *Culicoides variipennis* (later reassigned as *Culicoides sonorensis* (Holbrook *et al.*, 2000) and referred to from herein by that name). This species was found to be vastly more amenable to scientific research than *C. imicola* in South Africa (Carpenter *et al.*, 2006; Venter *et al.*, 2005), being almost twice as large in body size (Wirth & Jones, 1957).

Results produced during the late 1950 and 1960 demonstrated that following infection and an incubation period of at least 10 days at 23°C, *Culicoides* are capable of producing clinical BT by feeding on sheep (Foster *et al.*, 1963). While these studies were a substantial step forward in implicating *Culicoides* as the major BTV vector group, the continuing lack of techniques enabling the quantification of virus titre in the donor sheep meant that the viraemic period was targeted using only host pyrexia and clinical signs. Later studies characterised this relationship as being non-linear (Darpel *et al.*, 2007; Veronesi *et al.*, 2010; Veronesi *et al.*, 2005) and this may explain some of the discrepancies noted by the authors in transmission rates. Moreover, prior to titration, samples were stored at -70°F (approximately -15°C) which was later found to be highly detrimental to preservation of infectious BTV (Luedke & Jones, 1972), reducing virus infectivity by at least 90% (Mertens et al 2005). Finally, the authors themselves raised the issue that they were utilising a colony strain of *C. sonorensis* and hence the behaviour of field populations could vary substantially from their results (Foster *et al.*, 1963).

Bluetongue virus replication within *C. sonorensis* was later investigated in greater detail, using both intra-thoracically inoculated females and those fed upon blood/BTV mixtures through artificial membranes (Foster & Jones, 1979; Jochim & Jones, 1966). All *Culicoides* directly inoculated with BTV were found to support replication of the virus (confirmed by observation of cytopathic effect in a lamb kidney

cell-line), in contrast to similar studies carried out in mosquitoes using other arboviruses, in which salivary gland barriers preventing dissemination had been discovered (Hardy *et al.*, 1983; Jochim & Jones, 1966; Kramer *et al.*, 1981). This study also demonstrated long-term infection of individual *Culicoides* without apparent loss of infectivity over time. It was therefore inferred that, once infected, *Culicoides* would be capable of transmitting BTV for their entire adult life (a fact later confirmed in a number of other studies e.g. (Jennings & Mellor, 1987).

In a later study utilising membrane feeding, *Culicoides* were incubated and then homogenised in pools (Foster & Jones, 1979). The titre of BTV in each pool was inferred from lethality to embryonated hens' eggs (ECE) and it was assumed that if a female of *C. sonorensis* was capable of ingesting approximately 10^{-4} ml of blood, it would then ingest ~10^2 median chick embryo lethal doses (ELD_{50}) of BTV when feeding on blood containing a BTV titre of ~10^6 ELD_{50}/ml. Following the initial ingestion of a blood-meal, a decrease in BTV titre was observed from days 1 to 3, the 'eclipse phase', corresponding to the digestion, attachment, penetration and un-coating of BTV in the mid-gut cells, during which process the replication of BTV could not be detected.

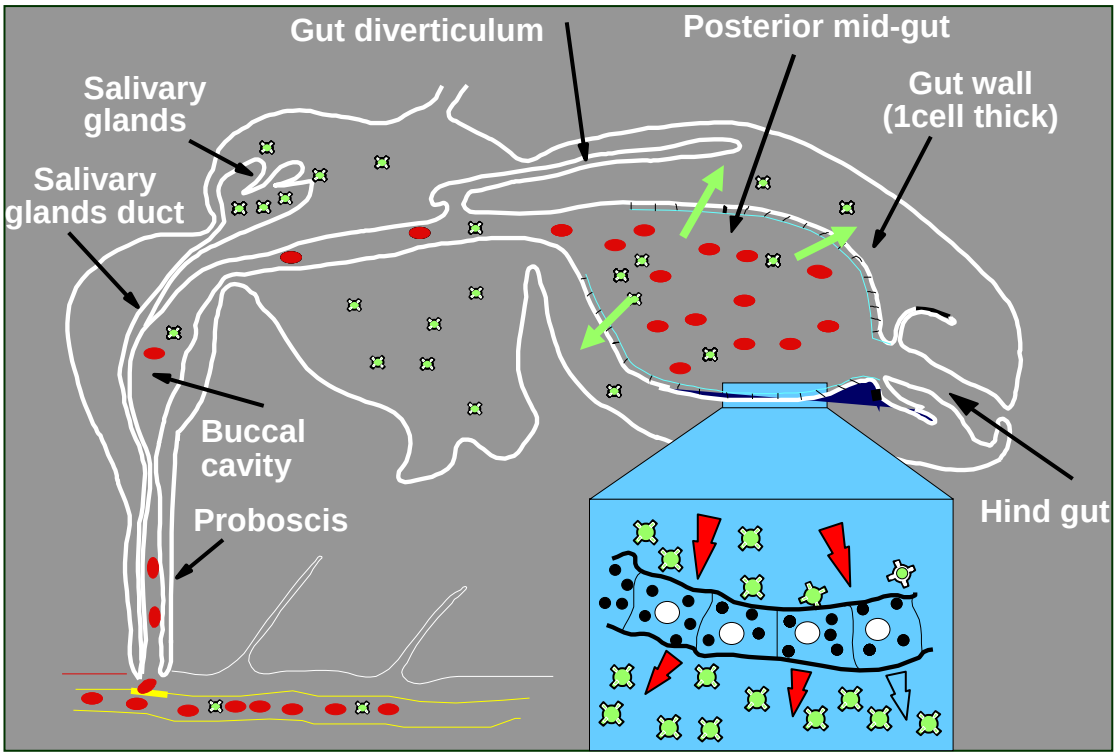

Figure 1.5: Dissemination of orbiviruses within a competent *Culicoides* following ingestion.
When female *Culicoides* bite a viraemic host, the virus within the blood meal is drawn into the proboscis and transported into the lumen of the hind part of the mid-gut, which is the primary site of infection. In the light blue square, details of the monolayer of mid-gut cells are shown: BTV particles (green) move from the mid-gut lumen to the haemocoel and eventually reach the salivary gland, where virus will continue to replicate and be ready to be inoculated with the following bite.
(Picture modified from Fu, 1995)

Following the eclipse phase, detectable BTV levels increased rapidly, reaching up to 10^5 ELD_{50} in some individuals by day ten post-infection (Foster & Jones, 1979). A second phase of replication, showing a further increase in virus titre of ~100-fold, then occurred from days ten to fourteen post-infection. This increase in BTV was thought to reflect the dissemination of the virus within the haemocoel and infection/replication in secondary target organs, including the salivary glands (Figure 1.5). The second plateau phase was suggested to represent either a "cessation of virus multiplication with retention of infectivity" or "a steady state of virus replication with its corresponding inactivation". Although it is still unclear which of these factors limits

the maximum titre of BTV developed, later studies demonstrated that smaller *Culicoides* species (such as *C. imicola* and *C. obsoletus* groups) develop lower maximum virus titres (Carpenter *et al.*, 2006; Jennings & Mellor, 1988; Venter *et al.*, 2005). It is also clear that although insects do not develop a circulating antibody response to viral infections, they do still have very effective immune mechanisms that can reduce or even eradicate infections, e.g by RNA silencing (Campbell *et al.*, 2008; Wang *et al.*, 2006; Zambon *et al.*, 2006).

1.2.2.2 Barriers preventing BTV dissemination

The replication and dissemination of BTV in orally infected *C. sonorensis* was later investigated in two separate studies using virus isolation and direct detection by immunofluorescence (Ballinger *et al.*, 1987; Chandler *et al.*, 1985). Virogenesis of BTV in the mid-gut and head of infected *Culicoides* was explored and showed that dissemination to these areas occurred only following 8-10 days incubation at 23°C (Chandler *et al.*, 1985). Although identification of BTV was successfully achieved in the head, the detection of particles in the salivary glands failed, possibly due to the presence of nonspecific staining. In addition, a lack of sensitivity in detection led to the recording of only limited dissemination of BTV to secondary organs e.g. abdominal ganglia and thoracic muscles which only rarely contained detectable BTV antigens. While preliminary in nature, this study did serve to point out the importance of defining dissemination of BTV in situ (Chandler *et al.*, 1985). A second study in the same laboratory examined dissemination, using only the immunofluorescence techniques and *C. sonorensis* orally infected with 3 different BTV serotypes (BTV-10, 11 and 17) (Ballinger *et al.*, 1987). Contrary to the previous experiments, BTV was detected in the head (>60% of individuals examined) and salivary gland (17%) after only 5 days,

although salivary gland infection was more common at day 14 post infection (>70%). This contradicted previous studies suggesting BTV replication in target organs only occurred from day 10 onwards (Foster & Jones, 1979). The maximum infection rate was achieved at day 14 and then remained stable to day 20 post-infection when the experiment was terminated (Ballinger *et al.*, 1987).

Parallel studies were carried out using *C. sonorensis* females derived from the same BTV-susceptible colony described above (named "Sonora 000") (Jones, 1957), that had been transferred to the Pirbright laboratory during the 1970 (Boorman, 1974). These studies used a BHK-21 detection system to show the presence of a 'threshold' level of virus infection after eight days post infection at 25±1°C, of 2.5-3.0 $\log_{10}TCID_{50}$ that was indicative of full dissemination of the virus in the insect (Jennings & Mellor, 1987). Below this threshold it was shown that the virus was completely restricted to mid-gut cells. It was concluded that a 'mesenteron escape barrier' (MEB) could prevent virus escape from the gut cells, preventing its dissemination via the haemocoel and reaching secondary target organs including the salivary glands (Mellor, 1990). However, above this threshold, full dissemination to the salivary glands had occurred, leading to the higher replication level detected, although the evidence confirming this was restricted to unpublished results.

Studies in the 1980 also explored vector-pathogen relationships, involving the effect of treating BTV with proteolytic enzymes (such as trypsin and chymotrypsin). It was suggested that these enzymes, which are known to be present in the mid-gut of *Culicoides*, might be involved in conversion of intact BTV virus particles to infectious sub-viral particles (ISVP) by cleavage of the outer capsid protein VP2 (Mertens *et al.*, 1986; Mertens *et al.*, 1987). The specific infectivity of ISVP for insect cell lines and adult *Culicoides* is much higher (by approximately 100 times) than that of the

unmodified virus particles (Mertens *et al.*, 1987; Mertens *et al.*, 1996). The presence of proteolytic enzymes in the vector's gut and saliva (Campbell *et al.*, 2005; Darpel *et al.*, 2011) suggests that the formation of ISVP plays a significant role in the mechanism of virus infection in the gut cells of *Culicoides* species.

During the 1990, the subject of BTV dissemination in *C. sonorensis* was revisited directly using immunohistochemistry (Fu, 1995; Fu *et al.*, 1999), contributing to a clearer understanding of the infection and dissemination process. In these studies, virus dissemination within *Culicoides* was identified as a rapid process that occurred as early as seven days after incubation of orally infected *C. sonorensis* at 25°C. This study also indicated that another barrier mechanism was in operation, that was identified as a 'dissemination barrier' (DB) associated with restriction of BTV to fat body cells. These cells had previously been found to be infected with BTV (Ballinger *et al.*, 1987), but were known to function as an immune mechanism in other insect species (Dimopoulos *et al.*, 1997; Hoffmann, 1995; 2003).

Other as yet undefined immune responses may be linked to the MIB and MEB, and could therefore play important roles in BTV infection, replication, dissemination and release within the vector insect. Of particular interest in this regard is the dissemination barrier, which may be dependent on immunological mechanism(s) of the insect that could inactivate or destroy the virus infectivity (e.g. RNA interference or silencing (Burand & Hunter, 2012)), rather than purely physical barriers. At present the immunological responses of *Culicoides* to BTV infection have not been investigated despite significant progress in work with mosquitoes (Blair *et al.*, 2000; Keene *et al.*, 2004; Sanchez-Vargas *et al.*, 2004).

1.2.2.3 Genetic and environmental effects influencing Culicoides *vector competence*

A key discovery among more recent studies was that vector competence of *C. sonorensis* was at least partially a heritable trait, inferred from a process of selective breeding and systematic testing of susceptibility to infection with single BTV strains (Holbrook *et al.*, 1996; Luedke *et al.*, 1976; Tabachnick, 1990; 1991; 1992; Tabachnick, 1996b). The underlying mechanism for this remains poorly defined (Mellor, 2004a), however, it was suggested that vector competence for infection with BTV in *C. sonorensis* is controlled by a single, maternally inherited, genetic locus (Tabachnick, 1991). The degree to which this relationship is representative of other *Culicoides* species transmitting BTV is not known.

Temperature is known to determine the length of the extrinsic incubation period (EIP) in adult *Culicoides* (the time between ingestion of infectious blood and transmission) (Mellor & Wittmann, 2002; Mullens *et al.*, 2004; Mullens *et al.*, 1995; Wittmann & Baylis, 2000; Wittmann *et al.*, 2002), as would be predicted from earlier studies of mosquitoes (Reeves *et al.*, 1994; Turell *et al.*, 1985). In addition, however, temperature was also found to significantly influence the overall vector-competence of *C. sonorensis* (Wittmann *et al.*, 2002). Higher incubation temperatures during the EIP, increased the proportion of *C. sonorensis* susceptible to infection with BTV, AHSV and EHDV. The virus replication rate in the vectors is thought to be largely dependent on the activity of the virus-core polymerase, which has a temperature optimum from 29-35^{o}C (Mertens, unpublished data; (Vandijk & Huismans, 1982). High ambient temperatures are therefore likely to increase the rate of virus replication and dissemination within *Culicoides* and may additionally lead to increases in barrier permeability within *C. sonorensis*.

High developmental temperatures have also been found to induce a higher level of vector competence in species that are usually regarded as 'non-vector'(Wittmann, 2000). This effect which was attributed to a mechanism commonly termed the 'leaky gut' phenomenon, initially identified in mosquitoes (Boorman, 1960; Hardy *et al.*, 1983). Full BTV dissemination was recorded in up to 10% of adult *C. nubeculosus*, following incubation as larvae at 33ºC, while the competence of midges incubated ata 'standard' temperature (Fu *et al.*, 1999) for the same strain of BTV was less than 1%. It was concluded that this represented a breakdown of the integrity of the gut wall, with subsequent leaking of the virus through the basal laminal cells of the mid-gut (Wittmann, 2000). This allowed the virus to bypass the mid-gut barriers and disseminate via the haemocoel, thus reaching the salivary glands. The relevance of these observations to the field situation is at present unclear, as the numbers of species consistently exposed to such temperatures in the field is probably limited to those in tropical regions utilising dung as a developmental substrate. These include both highly competent (e.g. *Culicoides bolitinos* in RSA (Meiswinkel *et al.*, 2004b; Venter *et al.*, 1998)) and refractory species (e.g. *C. brevitarsis* in Australia (Bishop *et al.*, 1996)).

The susceptibility of adult *Culicoides* to infection may also be affected by parasitism of individuals leading to physical disruption of gut barriers (although still based solely on laboratory observations) (Mellor & Boorman, 1980). Dual infection studies of *C. nubeculosus* using a blood mixture containing both BTV and *Onchocerca cervicalis*, showed a higher infection rate, as compared to an absence of infection in insects fed with blood and BTV only. Recent studies have also demonstrated the importance of gut microfauna on arbovirus infection of mosquitoes and have shown the reduction of RNA virus replication induced by bacterial endosymbiont (*Wolbachia*

pipientis) of *D.melanogaster* (Glaser & Meola, 2010; Walker *et al.*, 2011). These studies have not been carried out for *Culicoides*-arbovirus systems.

1.2.2.4 Vector competence testing using field collected Culicoides

From the preceding sections it is clear that our knowledge concerning the transmission of BTV by *Culicoides* is largely based upon a single species, *C. sonorensis*. The rearing and maintenance of additional *Culicoides* species has proved to be difficult due primarily to an inability to blood-feed field collected individuals, or elicit successful mating and subsequent survival of larvae in order to colonise other species (Boorman, 1985; Carpenter *et al.*, 2001; Veronesi *et al.*, 2009). While a large number of studies have been carried out on the colonisation of *Culicoides* (Carpenter, 2001; Carpenter *et al.*, 2001; Fahrner & Barthelmess, 1988; Hair & Turner, 1966; Hunt, 1994; Kettle *et al.*, 1975; Linley, 1968; Mullens & Schmidtmann, 1981; Mullens & Velten, 1994; Sun, 1969; Veronesi *et al.*, 2009), only two species are at present commonly reared under laboratory conditions.: *Culicoides nubeculosus* (Boorman, 1974; Downes, 1950; Megahed, 1956) and *Culicoides sonorensis* (Boorman, 1974; Jones, 1957),

In the absence of colonies of the *Culicoides* species that are considered as the primary 'potential' vectors, field-collected *Culicoides* have commonly been used in assessments of vector competence for arboviruses (Bonneau *et al.*, 2002a; Carpenter *et al.*, 2006; Standfast *et al.*, 1985; Venter *et al.*, 1998). This can be accomplished using two complementary methods. Firstly, circulation of BTV in local populations of *Culicoides* can be assessed through directly examining infections in field collected individuals (Nevill *et al.*, 1992a; Nevill *et al.*, 1992b). This technique has two major limitations in that the traps used to collect *Culicoides* populations for assessment of infection status rarely collect populations that are representative of biting individuals on

ruminant hosts and exposure of subjects to viraemic ruminants cannot be assumed. While the first of these limitations can be addressed through separate wide-scale surveillance and direct collection of *Culicoides* on hosts, information regarding proportional susceptibility to infection can only realistically be assessed by laboratory examination. In these studies, *Culicoides* are fed either upon live viraemic hosts (Foster *et al.*, 1968; Foster *et al.*, 1963) or, more commonly, through artificial feeding methods such as membrane-based oral infection (Davis *et al.*, 1983; Hunt & McKinnon, 1990). In these methods a blood/virus suspension is heated to 37°C to simulate host body temperature and feeding occurs through an artificial (e.g. Parafilm) or natural (e.g. chick skin) membrane (Davis *et al.*, 1983; Roberts, 1950; Venter *et al.*, 1991). Following the EIP, the proportion of infected individuals can be assessed using a variety of standard diagnostic techniques.

The power of combining these techniques has been demonstrated in studies of the vector competence of *C. imicola* and *C. bolitinos*. Primary transmission of BTV in South Africa has traditionally been attributed to *C. imicola* (Du Toit, 1944; Mellor *et al.*, 1984; Walker & Davies, 1971). In cooler areas bordering Lesotho, however, *C. imicola* was only present in low abundance, but BTV circulation was still detected. In these environments another morphologically similar species, *C. bolitinos*, was abundant (Nevill *et al.*, 1992b; Venter & Meiswinkel, 1994). Under laboratory conditions, this species was subsequently shown to be susceptible to infection at a rate approximately twenty times higher than *C. imicola* (Venter *et al.*, 1998; Venter *et al.*, 2011). This balance between the population abundance, distribution, susceptibility to infection and other variables affecting the ecology and behaviour of different vector species (e.g. entry into buildings) remains relatively poorly characterised.

The use of specific techniques for infection, processing and detection of infected individuals is largely driven by consideration of logistics and the behavioural malleability of the *Culicoides* species concerned. Methods used to detect the virus in adult *Culicoides* collected from BTV outbreak sites, have evolved in parallel with advances in more general diagnostics, from the injection of homogenates of putatively infected individuals directly into animal hosts (Du Toit, 1944), to the isolation of BTV in a variety of cell lines (Fernandes, 1959; Wechsler & McHolland, 1988) or ECE's (Alexander, 1947; Jones & Foster, 1966). Recently, roboticised real time RT-PCR (rtRT-PCR) assays that offer extremely high sensitivity and high-throughput capability have been used to confirm BTV RNA in field collected pools of *Culicoides* (Hoffmann *et al.*, 2009a; Shaw *et al.*, 2007; Toussaint *et al.*, 2007a). The changes in and diversity of detection methodologies poses significant difficulties in both standardising current studies and in interpreting historical detections of BTV in putative vectors. This issue, which is common in the history of studies of all vector groups, can be most easily illustrated by examining the progression of vector competence studies in northern Europe during the BTV-8 incursion (Carpenter *et al.*, 2008a; Carpenter *et al.*, 2009).

1.2.3 Studies of Culicoides *vector competence in Europe*

Culicoides imicola, which is thought to be the major sub-Saharan vector of BTV, has long been known to be present in southern Europe. Since the first outbreaks of BTV in Portugal, Spain and the Greek islands between 1956 and 1979 this species was consistently assigned as the main vector for BTV transmission (reviewed by (Boorman, 1986; Mellor *et al.*, 1983; Mellor & Wittmann, 2002). This assumption was initially also supported by the correlation of *C. imicola* distribution with BTV outbreaks area reviewed in (Mellor, 1990; Purse *et al.*, 2005). However, BTV-4 had also been

isolated from field collected *C. obsoletus* complex females in Cyprus (Mellor & Pitzolis, 1979).

Incursions of BTV into Europe from 1998 to 2005 were notable for the occurrence of disease outbreaks in areas where *C. imicola* was thought to be absent (above the 40° north parallel) (Boorman *et al.*, 1985; Caracappa *et al.*, 2003; Mellor *et al.*, 1983). A clear example of this was the BTV outbreaks in Bulgaria which occurred in an area far from the distribution of *C. imicola*, but where *C. obsoletus* group and *C. pulicaris* group species were highly abundant (Dilovski *et al.*, 1992; Georgiev *et al.*, 2001a; b). An identical situation was described in Italy by other authors (Savini *et al.*, 2003) where once again, BTV was circulating in areas where *C. imicola* was very scarce, while species of the *C. obsoletus* complex were most abundant. BTV was subsequently identified in *C. pulicaris* from Sicily (Caracappa *et al.*, 2003) both by detection of viral RNA using RT-PCR, but also by virus isolation in ECE's from non-blood engorged, parous, field collected females. Following this finding, additional studies confirmed virus presence in *C. obsoletus* complex individuals in other regions of Italy (De Liberato *et al.*, 2005; Ferrari *et al.*, 2005; Savini *et al.*, 2005). Variations in the susceptibility to infection among *C. obsoletus* group, *C. impunctatus and C. pulicaris* group were recorded in the laboratory using insects from the UK, outside the BTV outbreak regions (Carpenter *et al.*, 2006). Female orally infected with BTV, showed an infection rate of BTV between 0.4 - 7.4% after an incubation period of 7-10 days with a titre varying between 0.8-4.8 $\log_{10}TCID_{50}$/midge. According to levels previously recorded in *C. sonorensis* (Fu *et al.*, 1999; Jennings & Mellor, 1987), the threshold level indicating 'transmissible infection' ($\geq$2.7 $\log_{10}TCID_{50}$/midge) was also reached in the UK species, suggesting that BTV particles had managed to escape the dissemination

barriers and were therefore likely to have reached the salivary glands (Jennings & Mellor, 1988).

Since the Northern European BTV-8 outbreak in 2006, several studies were conducted to examine the vector-competence of adult *Culicoides* collected at outbreak sites (Dijkstra *et al.*, 2008; Mehlhorn *et al.*, 2007; Meiswinkel *et al.*, 2007). Large numbers of field samples were collected, and molecular diagnostic techniques able to process large volume of ruminant-derived samples were already readily available within the respective national reference laboratories. Although, none of these field collected *Culicoides* were processed for virus isolation, many were tested using real-time RT-PCR (rtRT-PCR) assays to detect the virus (or, more correctly to detect the presence of viral RNA) within pools of *Culicoides*. Despite the later inclusion of cycle threshold (C_t) values, as a proxy indication of RNA quantity (Hoffmann *et al.*, 2009a; Vanbinst *et al.*, 2009), several concerns still remain concerning the interpretation of 'positive' findings. For example, it is possible that BTV could have persisted in the adult *Culicoides* tested following ingestion of an infected blood meal, or could possibly have been detected due to a sub-transmissible infection that commonly occurs in other members of this genus (Mellor, 2000). One of the main difficulties in the interpretation of these findings and assigning vector competence to species of midges, was the use of unstandardised / unvalidated assays (Carpenter *et al.*, 2009).

1.2.4 Standardising assays for assessing vector competence.

To date, BTV transmission has largely been investigated on the basis of financial imperative (Carpenter, 2009; Gunn, 2008; Tabachnick, 1996a; Wilson & Mellor, 2009b). Consequently, major outbreaks of BTV in Europe have led to recent advances in this area. In much the same way the apparent emergence of BTV strains in

South Africa, the USA and Australia led to earlier detailed studies in those countries. However, our global knowledge concerning primary vector species, outside of these regions, remains poor (for reviews see (Carpenter *et al.*, 2006; Mellor *et al.*, 2009)). Despite the paucity of reliable data and the lack of comparability between studies using different technologies (as already discussed), it has been suggested that specific BTV strains may become adapted to certain *Culicoides* vector species /populations,forming stable virus-vector 'episystems' (Tabachnick, 2004).

The existence of two such systems has been described in the America, characterized by the presence of two different primary vectors for BTV: *C. sonorensis* in North America (Price & Hardy, 1954) and *C. insignis* in Central- South America (Tanya *et al.*, 1992). Briefly, it has been hypothesized that these two vectors, share only a small region of distribution overlap and that this leads to adaptation of BTV strains to these specific vectors. The main concept on which this theory is based, is the presence of as yet unknown mechanism and or/adaptations to a specific ecosystem by these BTV competent species.

The lack of vector competence in species phylogenetically closely related to *C. sonorensis* (such as *C. occidentalis* and *C. variipennis*) (Holbrook & Tabachnick, 1995; Tabachnick, 2004) suggests that these mechanisms may not be genetically related. However, the exact mechanisms that might be involved in episystem development are still not completely investigated, and there is no information to explain the possibility and consequences of the introduction of different strains of BTV into new regions.

It is self-evident that this view of stable systems may have a varying degree of validity, according to the specific episystem considered and in all cases relies on limited surveillance data. Recent detection of new and previously exotic BTV strains/serotypes in the USA, Europe and Australia suggest that existing episystems may break down

with changes in environmental conditions or the introduction of new strains (Maan *et al.*, 2012a). The *C. sonorensis*/*C. insignis* system in the USA, which was proposed as one of the most stable episystems, has seen the incursion of 10 new BTV serotypes into North America from South or Central America (MacLachlan, 2010; 2011; Ostlund, 2010). Interestingly, previous studies have shown that differences do occur in the sequence of NS3 from BTV strains circulating in different geographical areas of the United States. Moreover, viruses isolated from *C. sonorensis* circulating in a single site, showed significantly less NS3 heterogeneity, indicating what could represent a founder effect in virus replicating within the insect (Bonneau *et al.*, 2002b). The influence of significant changes in the BTV genome through genome segment exchange (reassortment), or more subtle effects caused by genetic drift, and their effect of virus-infectivity for a particular species of *Culicoides* have not been fully investigated. It is also uncertain that *Culicoides* species which were considered in each study, are the only ones capable of acting as vectors for BTV in each region, or that individual BTV strains cannot adapt to other *Culicoides* species.

In Europe the incursion of multiple strains of BTV has been characterised by variations in the location, degree and timing of their spread. As an example, the 'eastern' BTV-1 strain that was initially isolated in Greece, failed to spread to other countries in the same manner as the other 3 serotypes that appeared in the eastern part of the Mediterranean region (BTV-4, 9 and 16) (Mellor *et al.*, 2009; Purse *et al.*, 2005) (Saegerman *et al.*, 2008). In contrast the 'western' BTV-1 strain that appeared in North Africa in 2006, spread successfully to Italy, Iberia and southern France, finally reaching Normandy (Maan *et al.*, 2009; Wilson & Mellor, 2009b). It is currently unclear whether these observed differences are due to variation in adaptation of BTV strains to different *Culicoides* species, including Palaearctic *Culicoides species*, or whether other factors

(e.g. climate, host presence, temperature) can influence virus distribution (Balasuriya *et al.*, 2008; Wilson *et al.*, 2000).

A possible mechanism involved in BTV strain emergence is the exchange of genome segments between different BTV strains circulating in the same region ('re-assortment'). Following the advent of full genome sequencing of BTV strains, the presence of reassortants is increasingly being reported in European countries (Batten *et al.*, 2008b; Brenner *et al.*, 2010; Maan *et al.*, 2009). These strains have included a reassortant between BTV-16 and BTV-2 vaccine strains in sheep blood collected in the field. The authors describe a 're-assortant' virus containing genome segment 2 (VP2) derived from the BTV-16 vaccine (with a 99.8% nt identity) and genome segment 5 (NS1 gene) identical to the Seg-5 of BTV-2 vaccine. Another example of re-assortment has been observed in the BTV-16 strain from Cyprus (CYP2006/02) containing the NS1 gene from the BTV-1e strain previously detected earlier in Greece (Maan & Mertens personal communication). There is also evidence that the strain of BTV-9 from Bulgaria and Turkey 1999-2001, which is an eastern strain, contains a 'western' genome segment 5 (encoding NS1), which could only have been acquired by reassortment (Maan et al in prep.). Genetic drift and re-assortment of BTV genome segments, contributes to the genetic diversity and evolution of field strains of the virus, with the potential for emergence of more virulent strains (Bonneau *et al.*, 2001; Oberst *et al.*, 1987; Samal *et al.*, 1987a; Samal *et al.*, 1987b).

1.3 Aims and Objectives of the Current Project

The overall aim of this project is was to devise novel standardised methods of investigating BTV replication in both field collected and colonised *Culicoides* and to use these methods to explore what factors limit BTV strain occurrence and spread in Europe. Using these standardised laboratory techniques, this project will enhance our understanding of the virus- and vector-related factors that influence BTV distribution within Europe, helping us to understand the risks of further outbreaks and predict their occurrence.

The first aim of the project was to optimise and validate a high-throughput method for the quantification of virus in *Culicoides* infected orally or intrathoracically with BTV. A standardised and reproducible method that can be used to rapidly and accurately estimate viral load, in terms of the amount of infectious virus particles and/or viral RNA in individual or pooled *Culicoides*, would support valid cross-laboratory studies on vector competence. In this study, a Tissue Lyser method was optimized for the homogenization, isolation and quantification of BTV from individual or pooled adult female *Culicoides*. The efficiency of the Tissue Lyser was also compared to that using the standard grinding method (polypropylene motor-driven pestles).

The second aim of this project was to devise a methodology for testing BTV replication rates in insect cells (ABADRL-Cv-KC - *C. sonorensis* embryo cell line), including developing and standardising an Antigen Enzyme Linked Immunosorbent Assay (AgELISA). This will reduce the need for time consuming and highly selective diagnostic assays such as the use of inoculation into embryonated hens' eggs (ECE) or mammalian cell lines (BHK-21, VERO). The KC cell line has the advantage of a high sensitivity to BTV strains and is robust, so easily distributable to other laboratories. KC cells also replicate at a wide range of incubation temperatures.

The third aim of this project was to produce standardised methods for detecting the level of dissemination of BTV in infected *Culicoides* vectors. Based on previous studies, detection of virus in the insects' head implies a full disseminated infection, as this genus appears to lack a salivary gland barrier. It is hypothesised that replication within the cerebral ganglia and other organs in the head region would therefore indicate a fully disseminated infection and transmission competence. This conclusion was tested by exploring dissemination within several *Culicoides* species and in a variety of origins, to produce techniques that can be used worldwide to estimate vector competence. Finally, confocal microscopy of BTV dissemination in infected *C. sonorensis* was examined as a means of visualising vector competence.

The fourth aim of the project was to investigate methods to define the thermal replication-limits and EIP of different BTV strains in KC cells and adult *C. sonorensis*. In these studies, 12 BTV strains were screened for replication efficacy at different incubation temperatures. Replication will be detected using rtRT-PCR and BTV titration followed by AgELISA to define levels of infectious virus. Two strains were then selected for further study using *C. sonorensis* as a model host, with a view to defining whether thermal limits or efficiency of replication may in part determine spread patterns of BTV strains in Europe.

The fifth aim of this project was to investigate the genetic impact of adaptation of BTV to different cell lines. Recent studies on arboviruses transmitted by mosquitoes (e.g. Chikungunya virus, Dengue virus, Yellow fever virus, West Nile virus) (Ciota *et al.*, 2007a; Weaver *et al.*, 1999) have demonstrated that multiple passages in cell culture results in changes to the nucleotide sequence of the virus genome. It has been suggested that these changes can be related to alteration (attenuation) of the virulence characteristics of the virus and the vector competence for some mosquito vectors (Van

Slyke *et al.*, 2012). This genome sequence was examined for a BTV strain (BTV-8) that had been adapted (with different passage histories) to two different cell lines (BHK-21 and KC cells). Comparisons of nucleotide or amino acid changes and their impact on infection rate and dissemination in the vectors were also discussed. In addition, *D. melanogaster* will also be investigated as a novel model host for study of BTV replication at a molecular level.

Chapter 2: Materials and Methods

This chapter summarises standard materials and methods used throughout the project. Additional more specialised techniques associated with investigations are detailed within each chapter.

2.1 Cell Lines Used During the Studies

2.1.1 Mammalian Cell Line (BHK-21)

An established fibroblast cell line, derived from baby hamster kidney cells (BHK-21 clone 13 Glasgow 1962/3 (Stoker & Macpherson, 1964)), was used to propagate viruses for experimentation and as a means of detecting BTV in samples (Fernandes, 1959). This cell line is currently used by the Non-vesicular Disease reference laboratory (NVD-RefLab IAH – Pirbright, UK) in BTV growth and diagnosis and has historically been used in a wide variety of experiments involving BTV at IAH (Carpenter *et al.*, 2006; Jennings & Mellor, 1987; Veronesi *et al.*, 2005; Wittmann *et al.*, 2002). Growth media for BHK-21 cells contained L-glutamine Modified Eagles Medium (Glasgow-MEM) (Invitrogen) supplemented with, 10% foetal bovine serum (FBS) (mycoplasma / virus screened & BTV antibody free: GIBCO - Invitrogen). In addition, 1% antibiotic (1000 IU/ml penicillin/streptomycin) and 1% fungizone (4µg/ml Ampothericin B) were added. Maintenance media for BHK-21 cells was identical to growth media but excluded adult foetal serum.

During both growth and maintenance, BHK-21 cells were incubated at 37±1°C and 5% CO_2. BHK cells were grown in tissue culture flasks of different sizes and the amount of growth media used depended on the flask size (10ml for a 25cm^2 flask, 20-30ml for a 75 cm^2 flask and 50 ml for 175 cm^2 flask). For a 175 cm^2 flask, 80-90%

confluence is achieved within 1-3 days depending on the split-ratio. During passage, BHK-21 cell monolayers were removed by flash trypsinisation. The cell sheet monolayer was washed three times with 3 ml of EDTA-Trypsin (0.25%) (SIGMA), and then on the final wash incubated at 37±1°C and 5% CO_2 for 5-10 minutes. The BHK-21 cells removed from the flask surface were then harvested and resuspended at the desired split ratio (e.g. 1:10) into growth media and transferred to either new flasks (e.g. at a 'split' of 1:10) or 96 well microtitre plates (Nunc, UK). All manipulative experiments described below were carried out under a Microbiological Safety Cabinet (Class 2) or contained glove-box.

Strains of BTV maintained by the Arbovirus Molecular Research Group at IAH (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm) were used throughout the study (see Table 2.1 for description of strain origin, isolation method and storage method). According to the passage history or field isolate available (see Table 2.1), the amplification of each BTV strains was carried out by dilution 1:10 in appropriate media containing 1% antibiotic (1000 IU/ml penicillin/streptomycin) and 1% fungizone (4μg/ml Ampothericin B) ('diluent media'). The growth media was removed from a 175 cm^2 flask containing an approximately 90% confluent monolayer of BHK-21 cells. Four milliliters of diluted BTV were added to the flask and incubated on the monolayer for 15-20 min at room temperature (RT). Following incubation, 50 ml of new maintenance media were added to the flask, which was then transferred to an incubator maintained at +37±1°C and 5% CO_2 for 3-4 days. An untreated control flask with BHK-21 cells containing 'diluent media' only was treated in an identical fashion. The BHK-21 monolayers were observed every day and the supernatant from treatment flasks harvested when showing 100% cytopatic effect (CPE), taking into account aging of cells in the untreated control. The suspension of infected / lysed BHK-21 cells from

the treated flasks was then transferred into a plastic 20ml capacity universal tube and centrifuged for 10 minutes at 3,000 rpm to separate cells and debris. After centrifugation, the supernatant was transferred into a new labelled universal tube and stored at +4°C.

Amplification of virus from blood collected from a viraemic animal (cow or sheep) followed a different protocol. Blood was collected in Ethylenediaminetetraacetic acid (EDTA) to prevent its coagulation and transferred to a sterile plastic universal after removal of the plasma in suspension. The level of the blood was marked on the outside universal surface and sterile PBS (phosphate buffered saline) was added and mixed to provide a homogenous suspension. The suspension was centrifuged at approximately 3000 rpm (2400 g) for 5 to 10 minutes at ±4°C and washed and centrifuged three times. The packed red blood cells were then re-suspended with PBS up to a final volume equivalent to the original blood level marked on the tube. The washed blood was sonicated for 30 seconds at amplitude of 18 microns, to lyses the cells and release the virus, and then stored at +1°C to +8°C until required.

Table 2.1 Description of BTV strains used throughout the studies. This information are also listed in the dsRNA virus collection of IAH http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm within the Arbovirus Molecular Research Group.

IAH dsRNA virus collection number	**Virus Species & Serotype (identification method)**	**Place of original sample e.g. country town [grid reference] (date original sample was taken)**	**Isolated from which host species e.g cow, sheep, etc. (Date of virus isolation, where, by whom)**	**Original sample material; adapted to which cell types (Passage history of sample) [IAH passage history]**
BTV-9e KOS2001/03	BTV Type 9 (ELISA & SNT)	Municipality of Podujevo (30km north Pristina), village of Sllatina. (30/08/2001)	Sheep (Isolated at IAH Pirbright)	From blood sample [IAH/E1/BHK4]
BTV-9e KOS2001/02	BTV Type 9 (ELISA & SNT)	Municipality of Glogovac (35km west of Pristina), village of Sankovc (Kosovo) (12/09/2001)	Sheep (Isolated at IAH Pirbright)	From blood sample [IAH/E1/BHK1]
BTV-8w UKG2007/06	Confirmed as BTV-8 by Seg-2 serotype specific RT-PCR and sequencing	Collected from a rare breed farm, Baylham house, Baylham, Ipswich, Suffolk (UK)	Cow blood (female) from the highland cow called “Debbie”	Blood sample in PDAM
BTV-1w GIB2007/01	BTV-1 Identified as BTV-1 by type-specific RT-PCR targeting Seg-2	Gibraltar (5th Oct 2007)	Sheep (male). (Isolated at IAH Pirbright, October 2007)	[IAH/KC1]
BTV-4w SPA2004/02	BTV Type 4	Cadiz province at Jimena de la Frontera	Sheep (Isolated at IAH Pirbright)	Blood [IAH: E1/BHK1]
BTV-9w LIB2008/01	BTV-9 (group-specific rtRT-PCR & BTV-9 specific rtRT-PCR)	Libya	Blood from sheep with clinical signs of BT - Field sample	(IAH KC1)

IAH dsRNA virus collection number	**Virus Species & Serotype (identification method)**	**Place of original sample e.g. country town [grid reference] (date original sample was taken)**	**Isolated from which host species e.g cow, sheep, etc. (Date of virus isolation, where, by whom)**	**Original sample material; adapted to which cell types (Passage history of sample) [IAH passage history]**
BTV-9e BOS2002/02	BTVType 9 (ELISA & SNT, Seg-2 RT-PCR and sequence analysis)	Village: Milici Town: Vlasenica State/Province: Republike Srpska Country - Bosnia Herzegovina, (13-14/8/02)	Sheep (Isolated at IAH Pirbright 03/09/02)	BHK From blood (E1/BHK1) [IAH/BHK2]
BTV-2w SAD2002/02	BTV Type 2	Sardinia, Italy (2nd January 2002)	Sheep (Isolated at IAH Pirbright)	BHK cells (IAH/E1/BHK2)
BTV-16e ISR2009/01	BTV-16 (Seg-7 specific rtRT-PCR	Israel, from a village called: Ein Ha Tzeva (30th march 2009)	Sheep (Isolated at IAH Pirbright through KC cells)	KC2
BTV-16e SAD2004/02	BTV Type 16 (rtRT-PCR)	Sardinia, Italy (28th July 2004)	Sheep (Isolated at IAH Pirbright)	from frozen blood [IAH/BHK2]
BTV-1e GRE2001/01	BTV Type 1 (SNT & Seg-2 rtRT-PCR & sequencing)	Lesbos, Greece [39° 05´N, 26° 09´E] (2001)	Sheep 26/12/01	blood (BHK 21 cells) [IAH-BHK3]
BTV-8w NET2008/03	BTV-8	Staphorst region, Netherlands (7th August, 2008)	From cattle ~1yr old, unvaccinated animal clear clinical signs of BT, including fever and conjunctivitis	[IAH/KC1/BHK1] Derived from NET2008/01
BTV-1w FRA2007/18	BTV-1 (Seg-2sequence analysis)	France	From unvaccinated goat (Isolated at AFSSA- Maisons Alfort France in 2007)	[IAH/BHK1]
BTV-8w DEN2008/01	BTV-8 (LSI real time BTV 8 assay)	Denmark (29/8/09)	From cattle (Isolated at IAH-Pirbright),	[IAH/KC1]
BTV-4w MOR2009/09	BTV4 (Seg 2 rtRT-PCR)	Sheep sampled in Azrou Morocco on 28/10/09	Sheep isolated at IAH Pirbright	[IAH KC1]

IAH dsRNA virus collection number	**Virus Species & Serotype (identification method)**	**Place of original sample e.g. country town [grid reference] (date original sample was taken)**	**Isolated from which host species e.g cow, sheep, etc. (Date of virus isolation, where, by whom)**	**Original sample material; adapted to which cell types (Passage history of sample) [IAH passage history]**
BTV-4ww MOR2004/02	BTV Type 4 (Seg-7 group specific rtRT-PCR & Seg-2 rtRT-PCR)	El Kebir (north of Morocco)	Sheep	from blood [IAH-E1/BHK4]
BTV1e MOR2006/06	BTV Type 1 (Seg-7 group specific rtRT-PCR & serotype Seg-2 rtRT-PCR)	Isolated from a small village called Guenfouda near Oujda in the East of Morocco. (sample taken Oct 2006)	Isolated from a Sheep showing clinical BT in Morocco, using embyonated chicken eggs and BSR cells	(E1/BSR4) [IAH/BHK2]

2.1.2 Culicoides sonorensis *KC cell line*

The *C. sonorensis* embryonic KC cell line used during studies (Wechsler *et al.*, 1989) was provided originally by colleagues at the USDA lab in Laramie (European Collection of Animal cell Cultures [ECACC – 84100501]). Since these cells were supplied to IAH (late 1990), they have been passaged repeatedly at least 48 times. The KC cell line was grown in 'growth media': Schneider's Drosophila Medium (Gibco™), supplemented with 1% (or 2%) antibiotics (1000 IU/ml penicillin/streptomycin), 1% (or 2%) fungizone (4µg/ml Ampothericin B) and 12% foetal bovine serum (FBS).

Unlike the BHK-21 line, the KC cell line does not form monolayers and is maintained at cooler temperatures (25±1°C) without additional CO_2. As described for BHK-21 cells under 2.1.1, KC cells are grown in different size tissue culture flasks using the appropriate amount of growth media for the flask size. At an incubation temperature of 25±1°C, 6-7 days were required for multiple layers of KC cells to become confluent in a 175 cm^2 flask using a 1:10 split ratio. During passage, the media from the seed flask was discarded, and the appropriate amount of fresh Schneider's growth media was added. Cells were then detached from the surface of the flask by adding fresh Schneider's media 'diluent' (supplemented with 1% antibiotics and 1% fungizone as described above but without FBS), shaking and flushing through a 10 ml pipette then transferred in an appropriate dilution to a new flask containing Schneider's growth media. Flasks were passaged every 2-3 weeks according to the condition of the maintenance media.

Virus infection of KC cells was carried out using freshly passaged KC cells still in suspension. KC cells were passaged as described above; e.g. for a 175 cm^2 flask, 4 ml of 1:10 dilution of virus supernatant were added to the 50 ml KC cell growth media cell suspension and incubated for 7 days at +25±1°C . After the incubation period, cells were detached from the flasks by shaking.Supernatant and cells from the flask were harvested

into appropriate sized falcon tubes, centrifuged at 3,000 RPM to separate cells and debris and stored at +4°C until further use.

2.2 *Culicoides* Infection Methods Used During the Studies

The primary species used in these studies were *C. sonorensis* of the PIRB-s-3 strain which were originally derived from the American Sonora line (Jones, 1957), and *C. nubeculosus* line, originally colonised in the UK during the late 1970. Both lines have been maintained at IAH – Pirbright laboratories (UK) since using standardized techniques (Boorman, 1974; Wellby *et al.*, 1996). *Culicoides imicola* was also utilised in studies using field populations collected in the grounds of the Onderstepoort Veterinary Institute in the Republic of South Africa (25º29'S, 28º11'E, 1219 m a.s.l.).

2.2.1 Oral infection of C. sonorensis *with BTV*

Groups of approximately 250 adult *C. sonorensis* (2-3 days old) were held prior to blood-feeding in netted, waxed, card pillboxes (Watkins and Doncaster, Stainton, UK) (Fig.2.1d). The biting midges were deprived of food (5% sucrose solution) for 24 hours prior to being offered a blood meal. Blood-feeding was conducted using the Hemotek system (Discovery Workshops, Accrington, UK) equipped with a Parafilm® membrane (Cole-Parmer, Hanwell, UK) (Figures 2.1b&c). A metal blood meal holder disc, which functioned as the blood meal container (Figure 2.1a), was sealed with a membrane held in place with a rubber ring. Once sealed, a mixture of defibrinated horse blood (TCS Biosciences Ltd, UK) and BTV (4 ml of virus and blood 1:2) was poured inside through two holes present on holder. Horse blood was chosen instead of sheep blood, in order to avoid the presence of possible BTV antibodies. Two plastic lids were then used to seal the unit. The blood feeder was then screwed to a disc holder connected to the heater

device which maintained the blood meal temperature at a constant 37±1ºC. The membrane feeding unit was then placed on the top of the netted pillbox (Figure 2.1 d) containing the *C. sonorensis* which subsequently fed through the net and the membrane.

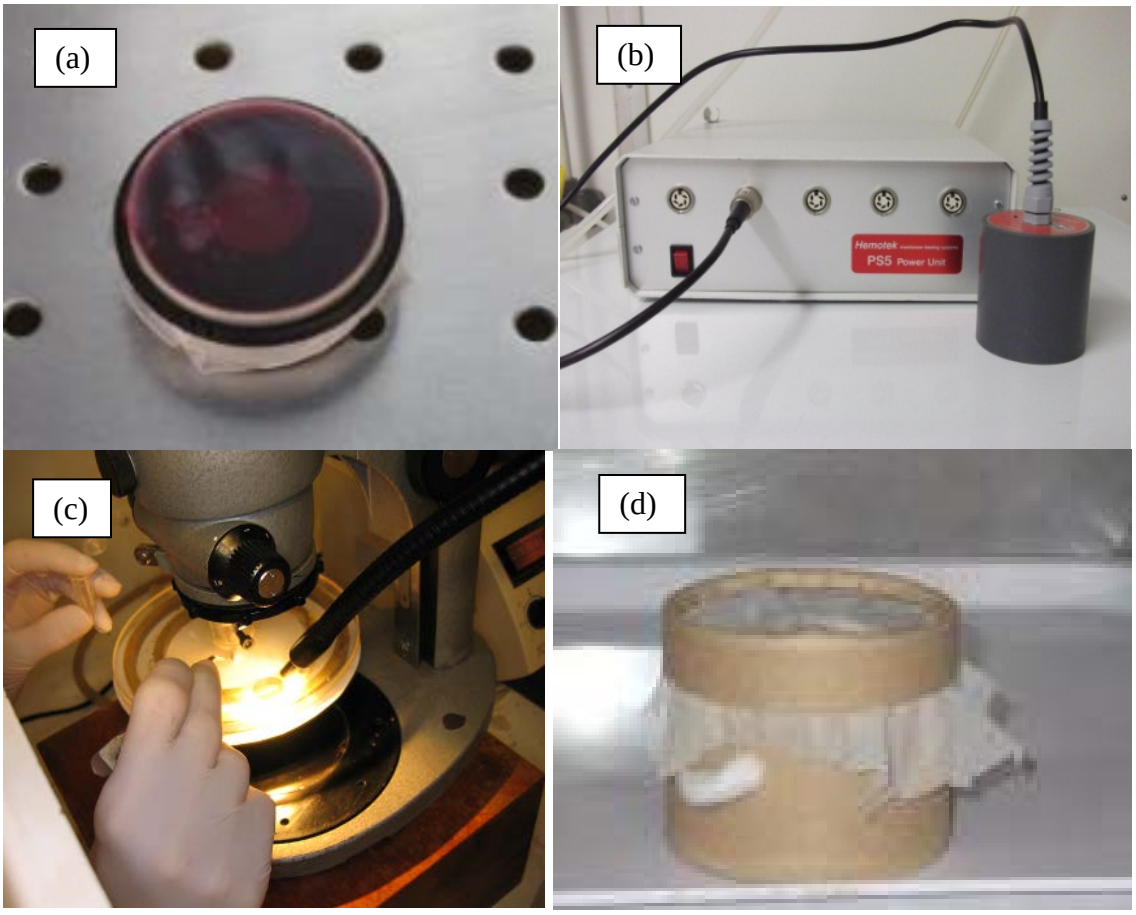

Figure 2.1: (a) Blood container filled with ~4 ml of infectious 1:2 blood mixture (horse blood and BTV); (b) Hemotek heating device unit; **(c) Stereomicroscope with rotating brass turn table containing adult *Culicoides* anaesthetised with CO_2 to be sorted and collected; (d) Waxed pillbox containing *C. sonorensis*.**

After approximately 30 minutes exposure the *C. sonorensis* inside the pillbox were immobilized using CO_2 and transferred to a rotating turn table under the stereomicroscope (Figure 2.1 c) with also intermittent release of CO_2 when further immobilization of he *Culicoides* was needed. Fully engorged females were identified using a stereomicroscope, and then transferred to a new pillbox container.

2.2.2 Oral infection of C. imicola *with BTV*

Insects attracted to light were collected using a down-draft, 220V OVI design light/suction traps equipped with 8W UV-light tube and run from 4 p.m. to 6 a.m. the following morning. Collected *Culicoides* were transferred to disposable unwaxed 250 ml paper cups covered with fine netting and stored at 25 ±1ºC and 85% relative humidity (Venter *et al.*, 1998). A cotton-wool pad moistened with a 10% (w/v) sucrose solution containing antibiotics (500 IU penicillin, 500 mg streptomycin and 1.25 mg/ml of fungizone) was provided daily. This food source was removed at 24-48 h post-collection and midges fed 24 h later for approximately 30 min on defibrinated sheep blood/BTV strain mixture through a chicken-skin membrane using a previously defined system (Venter *et al.*, 1991; Venter *et al.*, 1998) (Fig. 2.2). During feeding, lighting in the room was dimmed to approximately 1% daylight (65lux). Following feeding, *Culicoides* were immobilized in a freezer and blood engorged *C. imicola* females were selected on a refrigerated chill table and transferred to a 250 ml unwaxed paper cup (50 females/cup).

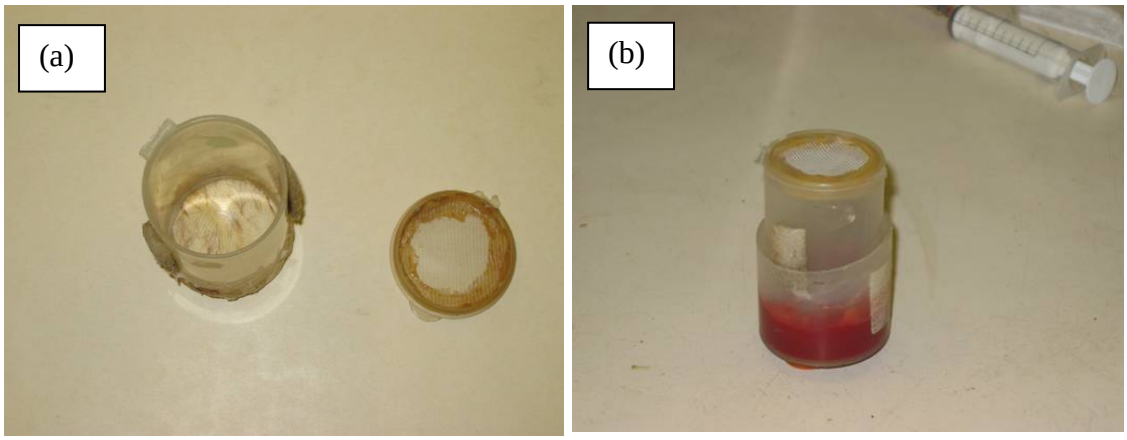

Figure 2.2: (a) Chicken-skin membrane feeder with netted lid; (b) Blood container with infectious blood mixtures and membrane feeder inserted

2.2.3 Intrathoracic (IT) inoculation of Culicoides

Inoculation of *Culicoides* was carried out at IAH, UK using tissue cultured BTV strains. Prior to inoculation, fine Pyrex glass needles were produced from capillary tubes (Narishige GDC-1, Narishighe International Limited) using a vertical Narishige needle puller (PC-10). A glass needle was then inserted into the needle holder of a microinjector instrument (Sutter Instrument Company, US) (Figure 2.3). A manual syringe driver for pneumatic or hydraulic control of injection needle was used for inoculation. Injection was controlled by the revolutions of a manual micrometre rotatable device (Figure 2.3). For each revolution, the plunger moves towards the tip of the syringe where the glass needle sits on the needle holder. The pressure applied to the oil by the movement of the plunger pushes the virus solution in the glass needle, resulting in ejection of the virus solution into the subject. The volume ejected can be controlling by moving the rotatable device.

Prior to injection, the insects were first immobilised with CO_2 and transferred to a brass turntable under a stereomicroscope (Figure 2.1c). Individual *Culicoides* were inoculated intra-thoracically with appropriate amount of virus preparation at the required titre. Inoculated insects were transferred either to a new pillbox and incubated for defined period of time or to a 1.5 ml Eppendorf tube for immediate processing.

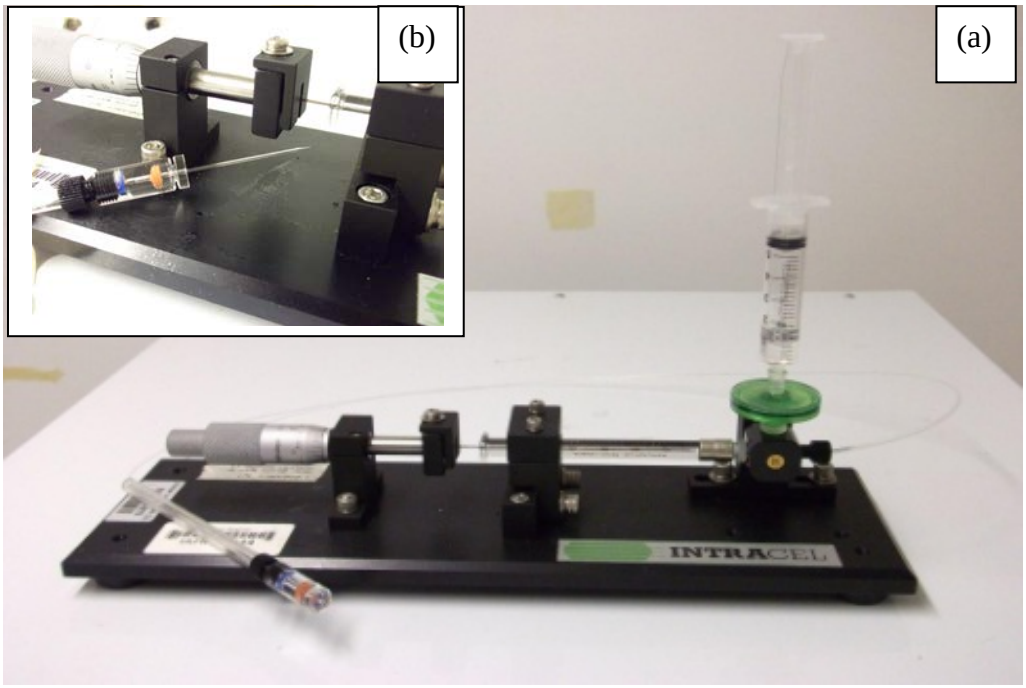

Figure 2.3: (a) Microinjector used for intrathoracic inoculation in *Culicoides;* (b) detail of the needle holder with glass needle inserted in it.

2.3 Preparation of *Culicoides* for detection of BTV

Following incubation at a range of temperatures and time periods specific to the study concerned a range of techniques were applied to determine BTV presence and dissemination within infected *Culicoides*. Most individual *Culicoides* were homogenised using two different methods; 1) a conventional grinding method, with a polypropylene, motor-driven pestle (Kimble, Kontes – Sigma Aldrich) using a standard Eppendorf 1.5 ml tube as the mortar; 2) using a Tissue Lyser machine manufactured by Qiagen (Crawley, UK). The standardisation of these methods was validated in Chapter 3. In the remaining *Culicoides*, BTV was detected through eliciting salivation, direct dissection or mounting for immunolabelling analysis.

2.3.1 Saliva collection from Culicoides

Salivation in *Culicoides* was induced by applying a pilocarpine (parasympathomimetic alkaloid) solution directly to the ventral surface. A solution

(solution 'A') was prepared by diluting 100 mg of commercial pilocarpine hydrocloride powder (Sigma – Aldrich) into 1ml of non-filtered deionised water. A second solution (solution 'B') was prepared by diluting 250 µl of solution 'A' into 750 µl PBS (supplemented with 0.1% tween-80). *Culicoides* were first immobilized with CO_2 and then fixed onto masking tape with their ventral surface exposed. A drop of approximately 0.1µl volume of solution 'B' was applied by using a 1 µl microcapillary glass tube (Microcaps – Drummond Scientific) to the ventral side of the abdomen of each immobilized female. *Culicoides* began to salivate after approximately ten minutes and saliva was collected in a 1 µl microcapillary glass tube (Microcaps – Drummond Scientific) previously filled with 10% FBS Glasgow's media. *Culicoides* proboscis was inserted into the glass capillary. *Culicoides* were allowed to spit into the capillary tube for approximately one minute and the media was then expelled into individual Eppendorf tubes containing 1ml of Schneider's 'diluent' media. These final solutions were stored at +4ºC until further use.

2.3.2 Dissection of adult Culicoides

Following incubation, adult female *Culicoides* were individually transferred onto a single cavity microscope slide (1.0-1.2mm thick 76x26mm) (Smith Scientific Ltd., UK) containing a drop of PBS. Sterile needles (Monoject™ Hypodermic Needles, 18 g x 1.5'') mounted on two sterile 1ml syringes, were used to dissect each *Culicoides*. Head, thorax and abdomen from each female were seperated and transferred into individual 1.5 ml sterile eppendorfs containing 100µl of Schneider's growth media with 2% antibiotics (1000 IU/ml penicillin/streptomycin), and 2% fungizone (4µg/ml Ampothericin B). Heads were homogenised using two clean glass cover slips, then transferred into the 1.5

ml eppendorf as described above. Each dissected body section was homogenised using a Tissue Lyser following the method described in Chapter 3.

2.3.3 Slide mounting of C. sonorensis *for confocal microscopy*

Culicoides sonorensis were prepared for immunolabelling by submersion in 2% paraformaldehyde for 48 hours at room temperature. The paraformaldehyde was then removed and *Culicoides* transferred into 12% sucrose in PBS. After overnight incubation at +4ºC, midges were washed in PBS and wings and legs removed. The rest of the body was transferred into a rubber mould (Stepped Microtome Embedding Mold – Embedding Mold-Dykstra - Science Services GmbH) (Figure 2.4) containing O.C.T (O.C.T. compound for microscopical cryotomy – BDH lab supplies). The mould was placed on dry ice for about 20-30 minutes after which it was immediately transferred to -80 degrees until used.

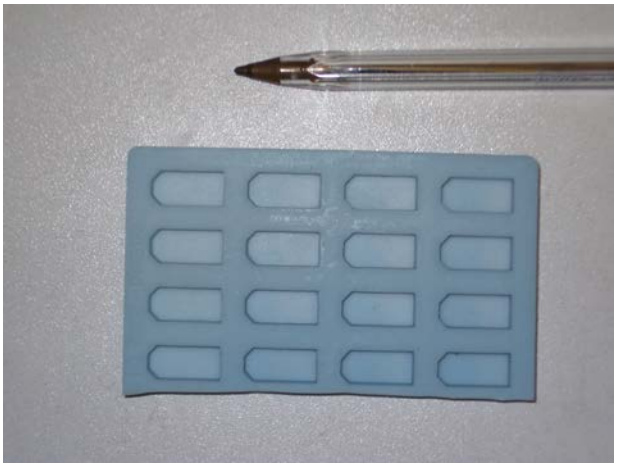

Figure 2.4: Rubber mould (Stepped Microtome Embedding Mold – Embedding Mold-Dykstra - Science Services GmbH) that was filled with O.C.T. emulsion (O.C.T. compound for microscopical cryotomy – BDH lab supplies) used to solidify adult *Culicoides* prior cryosectioning for Immunolabelling preparation.

The solidified O.C.T containing the midge was removed from the rubber mould and transferred to a holder block in a cryocutter instrument. The whole insect body was longitudinally cut (10µm thick sliced) and transferred to a polarized slide (2 slices per

slide). Each *Culicoides* slice was delineated with a hydrophobic barrier pen (ImmEdge™ Pen) to create a thick edge around it.

2.3.4 Immunolabelling and Visualisation of BTV in Culicoides

Slide mounted midge slices were incubated with PBS supplemented with 0.5% of BSA (Bovine Serum Albumine) for 30' at RT. The PBS/BSA was discarded and primary antibody added (polyclonal rabbit antibody 'Orab1' anti-NS2 diluted 1:1000 into PBS/BSA [supplemented with 0.05% Tween-20]). Slides were incubated for 45' at RT, the PBS/BSA/antibody was then discarded and slides were washed 4 times with Ca-Mg-free PBS. The secondary antibody (Goat anti-rabbit IgG conjugated to fluorescence Alexa Fluor® 488 – green colour) was diluted in the same PBS (1:1000) then added to the slide and incubated for 45 minutes at RT. Slides were washed in Ca-Mg- free PBS 4 times. Phalloidin (actin) (labelled with fluorescent Alexa Fluor® 568 - red colour) (dilution 1:25 in PBS) was added and incubated for 5 minutes at RT. After a further four washes in Ca-Mg- free PBS, DAPI-solution (dilution 1:10000 in filtered deionised water) was added. Slides were incubated for 10 minutes at room temperature and then washed four times in filtered deionised water, dried and mounted with Vectashield (Vector Laboratories) and cover slips (Agar Scientific). Slides were sealed with nail varnish and kept at +4ºC until ready to use for confocal microscopy observations. Slides containing immunolabelled body slices of infected *Culicoides* were observed by confocal microscopy in cooperation with the Bioimaging department (Dr P. Monaghan, Dr Pippa Hawes and Miss J. Simpson) at Pirbright laboratory (IAH, UK).

2.4 Detection and quantification of BTV

2.4.1 BTV detection and quantification in samples using BHK-21 cells

BTV detection based on cytopatic effect (CPE) observation in BHK-21 cells was carried out in 96 well microtitre plates (Nunc, Fisher Scientific, UK). One hundred microlitres of BHK-21 cell suspension in Glasgow growth media (2.1.1) was added to each well using a trough and multichannel pipette, containing approximately $2x10^4$cells/well. Plates were then stacked and sealed using cling-film with a cover placed over the top plate and incubated overnight at 37±1°C and 5% CO_2. The following day, a near-confluent monolayer of cells was observed (approximately 80-90% coverage). Growth media was then discarded from plates and replaced with 100 µl/well Glasgow maintenance media in all plate wells using a multichannel pipette and trough.

The degree of serial dilution of samples to be tested was dependent upon the expected likelihood of containing BTV. Homogenates of putatively infected *Culicoides* (1 adult/ml), for example, were initially tested at neat and 10^{-1} dilutions as a large number of samples were likely to test negative. All *Culicoides* samples that gave an initial positive result, were then titrated in a further dilution series (from 10^{-2} to 10^{-4}). For the dilution series, 900 µl of GMEM 'diluent media' (2.1.1) were transferred on a 5 ml glass bijoux and 100 µl of the sample to be diluted were added. From this 10^{-1} dilution, an aliquot of 100 µl was then transferred on a second glass bijoux containing also 900 µl of 'diluent media' to generate a 10^{-2} dilution and so on up to the required dilution fold.

Later in this thesis, dilution of samples was not carried out using glass bijoux but each dilution was performed on a deep 96 polypropylene well plate (Fisher Scientific, UK**)** with a capacity volume of 1ml/well. This allowed speeding up of the dilution process and also reduced the risk of bacterial contamination while opening and closing the bijoux frequently. One hundred microlitres of each diluted sample was then added to

each well using four replicate wells / sample dilution. A dilution series of a BTV infected BHK-21 cell supernatant was also titrated as a positive control. Plates were sealed, incubated at 37±1°C and 5% CO_2 and examined for CPE on days 3, 5 and 7 days post-infection (d.p.i.). Record sheets were scored as + for CPE and – for no CPE for each well. At 7 d.p.i. the titre of each sample was calculated as a 50% end point and expressed as $\log_{10}TCID_{50}$/ml, calculated using the Spearman-Karber formula (Finney, 1964) (see Appendix 8).

The presence of the BTV in test samples was also confirmed using a modified version of previously published indirect sandwich ELISA (Hamblin *et al.*, 1991; Thevasagayam *et al.*, 1996). Polyclonal rabbit antibodies [CH72, IAH Pirbright] (anti purified BTV-1 ref. strain RSArrrr/01) were freeze-thawed, dissolved in ELISA coating buffer solution (0.1 M carbonate/bicarbonate buffer, pH 9.6) (at 1:1000) and loaded (50 µl/well) on U well flexible polyvinyl chloride microtitre plates (Dynex Technologies). After overnight incubation of the plates at +4°C, the buffer was discarded and plates washed 3 times with PBS. The supernatant fluids from virus titration plate, were transferred into the pre-coated ELISA plates (50 µl /well). Plates were covered with plastic lids and placed in an orbital shaker rotating at between 100 and 140 rpm for 1 hr at 37°C. The plates were washed 3 times with PBS then 50 µl/well of polyclonal guinea-pig antibodies [CH73, IAH Pirbright] (anti-VP7), previously freeze-thawed and dissolved in blocking buffer (PBS containing 0.05% tween 20 and 5% skimmed milk powder) (at 1:1000), were added to each well. After orbital shaking for 45 min at 37°C, plates were washed 3 times in PBS, then 50 µl of polyclonal rabbit anti-guinea pig immunoglobulins/HRS (DAKO, UK, Ltd.) (diluted 1:1000 in blocking buffer) were added to each well and incubated for 45 min with shaking as described above. Plates were washed 3 times with PBS then 50 µl/well of substrate (0.4 mg/ml o-

Phenylenediamine dihydrochloride (Sigma, UK) and 0.05% hydrogen peroxidase solution) were added and plates incubated without a lid for 10 min at room temperature. To stop the reaction, 50 µl of 1M sulphuric acid was added per well. Plates were read using a spectrophotometer at a wavelength of 492 nm. Individual samples were recorded as positive if they gave an optical density (OD) >0.15 over at least 3 consecutive dilutions titrated from the strongest to the weakest dilution. Virus titres were determined as described in 2.4.1 (Finney, 1964).

2.4.2 BTV detection and quantification in samples using KC cells

BTV detection and quantification in KC cells was carried out using the same 96 well cell culture plates described for BHK-21 cells (Nunc, Fisher Scientific, UK). One hundred microliters of KC cells in Schneider's growth media suspension (obtained as described under 2.1.2) were added to each well. Sample dilution was approached as for BHK-21 CPE detection (2.4.1), but using Schneider's 'diluents media' (2.1.2) instead of GMEM's. After infection, plates were incubated at 25±1°C for 7 days. As BTV infection does not cause CPE in KC cells viral detection and quantification after incubation was carried out using secondary techniques, either in the form of an Antigen ELISA or by transferring KC cells titration plate supernatants onto BHK-21 cells (Figure 2.5) (see also Chapter 4 of this manuscript for more details on this subject). If detection relied upon transfer to BHK-21 cells, supernatant from each well (100µl/well) was transferred following incubation to a 96 wells cell culture plates containing a confluent monolayer of BHK-21 cells in GMEM maintenance media (100µl/well) (Figure 2.5). BHK-21 cells were then incubated at 35±1°C with 5% CO_2 for 7 days and checked for CPE at 3, 5 and 7 d.p.i. Viral titre was determined as described under section 2.4.1 (Finney, 1964) (see also Appendix 8). The presence of BTV in samples tested on KC cells was alternatively

confirmed using a modified version of a previously published indirect sandwich ELISA (Hamblin *et al.*, 1991; Thevasagayam *et al.*, 1996). The validation of this AgELISA test was part of the current thesis and it is fully described in Chapter 4.

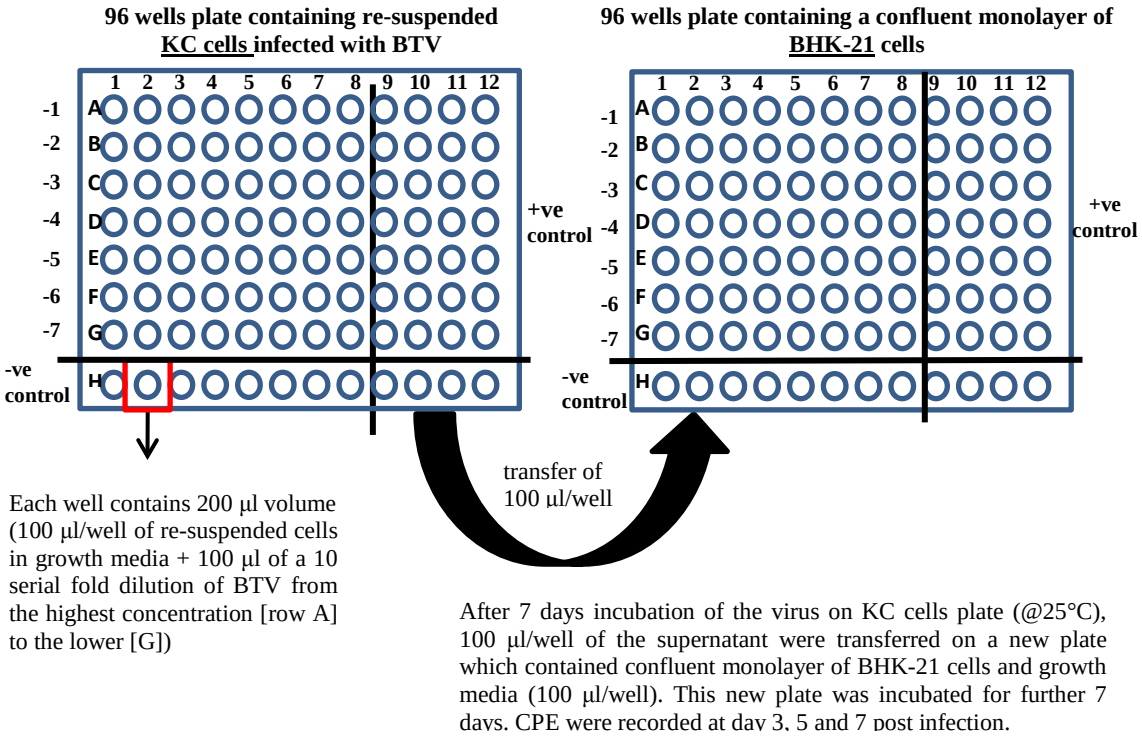

Figure 2.5: Scheme of BHK-21 cells infection with samples previously titrated on KC cells. A BTV dilution series (from 10^{-1} to 10^{-7}) (100µl/well) was incubated for 7 days at +25±1°C on a plate containing re-suspended KC cells (100µl/well). After the incubation period, supernatant of each KC cells (100µl/well) was transferred on a plate containing a confluent monolayer of BHK-21 cells (100µl/well). Cells were incubated at 35±1°C with 5%CO_2 for 7 days and checked for CPE at 3, 5 and 7 d.p.i.

2.5 Analysis of BTV strains

2.5.1 RNA extraction

Extraction of viral RNA was carried out using two different techniques: *QIAamp® Viral RNA mini kit, (Qiagen)* designed for cell-free fluid but also used here for extraction from the whole blood, infectious cell's supernatant (both KC or BHK-21) and *Culicoides'* homogenates, and by *96-well Universal BioRobot® (Qiagen)* used for infectious cell supernatant (KC or BHK-21 lines) and *Culicoides* homogenates. The choice between extraction by QIAamp Viral RNA mini kit and the Universal BioRobot was mainly driven by the total amount of samples to run as the robot is able to extract up to 96 samples/run (2 hrs) while manual processing is limited to 20 samples/hour.

QIAamp® Viral RNA Mini Kit (Qiagen, Crawley, UK): RNA extraction followed the manufacturers' instructions. Briefly, samples were lysed by placing 140 µl (double amount if using whole blood) of sample into a 1.5 ml tube containing 560 µl of AVL buffer, then mixing by pulse vortexing and incubated at ambient room temperature for 10 min. After incubation, 560 µl of ethanol (96%) was added to the samples which were vortexed and transferred to a spin-column. Since the capacity of the spin columns is 630 µl, samples were loaded in two steps. Once the RNA had been bound, the column was washed with 500 µl of AW1 buffer, and then centrifuged using a model 5217C centrifuge (Eppendorf AG, Hamburg, Germany) for 1 min at 8,000 rpm. A second wash was carried out with 500 µl of AW2 buffer for 3 minutes at 14,000 rpm. Finally, the spin column containing RNA was placed in a new 1.5 ml centrifuge tube and eluted in 40 µl of RNA free water, spinning for 1 min at 8000 rpm. The eluted RNA was then stored at -20°C until required.

96-well Universal BioRobot®(Qiagen, Crawley, UK): Prior to extraction, test samples were loaded into a deep-well s-block (QIAamp® All Nucleic Acid Kit MDx Kit

Qiagen, Crawley, UK). A 40µl aliquot of reconstituted protease in 6 ml of protease solvent was loaded into appropriate wells on the s-block followed by 50 µl of the test sample. A sample of previously defined quantity of BTV RNA (either as BTV-positive blood or tissue culture supernatant) was included in the extraction as a positive control, while negative tissue culture supernatant or RNA free water served as negative controls. Each full extraction plate contained at least 2 replicates of negative and positive control samples. Nuclease free water (190 µl) followed by 360 µl of lysis/binding buffer (MagNa pure LC total Nucleic Acid Isolation Kit (Roche diagnostic, Lewes, UK) were then added into each well, and the plates stored overnight, at +4ºC until ready for RNA extraction process. The extraction protocol with the Universal BioRobot® (Qiagen) using the QIAsoft software (QIAsoft 5.0) was developed by the NVD reference laboratory for their ISO 17025 accredited diagnostic assay for BTV and AHSV detection and is part of the SOP employed by the NVD reference laboratory. RNA was extracted within approximately 2 hours for a full 96 well plate. After the extraction programme was completed, the elution plate was stored at 4ºC (for few days) or at -20C (for weeks) until used for real-time RT-PCR.

2.5.2 BTV quantification by real time RT-PCR (rtRT-PCR)

The quantity of BTV RNA in *Culicoides* homogenates was evaluated using a modified version of the rtRT-PCR assay previously developed (Shaw *et al.*, 2007). To summarize, each reaction containing 6 µl of denaturated RNA sample, was heated for 5 min at 95°C in a PCR reaction plate, using a Eppendorf Mastercycle DNA Engine Thermal Cycle PCR instrument (Scientific Support US). Then 19 µl of rRT-PCR mastermix using the SuperScript® III Platinum®One-Step qRT-PCR Kit (Invitrogen) was added which contained:

- 12.5 µl of 2x reaction buffer mix,
- 1 µl of 20 pmols genotype-specific primers

  (eastern RSA - BTVrsa 291-311F GCGTTCGAAGTTTACATCAAT ;

  BTVrsa 387-357R CAGTCATCTCTCTAGACACTCTATAATTACG,

  western UNI - BTVuni 291-311F GCTTTTGAGGTGTACGTGAAC;

  BTVuni 381-357R TCTCCCTTGAAACTCTATAATTACG);
- 0.5 µl of 2.5 pmols specific BTVrsaProRedunR probe (3' –CYG GAT CAA GTT CAC TCC AYG GC -5');
- 0.5 µl Mg_2SO_4
- 0.5 µl 1:10 diluted ROX reference dye;
- 0.5 µl of the Superscript III/Platinum *Taq* enzyme mix (Invitrogen, Paisley, UK).

Reactions were loaded into a 96-well non-skirted polypropylene PCR plate for use with a Mx3005P PCR machine Stratagene® (Agilent Technologies, Stockport, UK) and capped using Mx3000P/Mx3005P optical strip caps (Stratagene®, Agilent Technologies, Stockport, UK). Plates were then placed in a Stratagene® Mx3005P PCR machine (Agilent Technologies, Stockport, UK) and amplification carried out under the following conditions: 55ºC for 30 minutes, one cycle (reverse transcription), 95ºC for 10 minutes, one cycle (denaturation of the Superscript III and activation of the Platinum *Taq* DNA polymerase), and 50 cycles of 95ºC for 15 seconds and 60ºC for one minute (Shaw *et al.*, 2007). The quantity of BTV RNA in the sample was assessed by the number of cycles required to give a threshold level of fluorescence (the cycle threshold value: C_t). Fluorescence was measured at the end of the 60°C annealing/extension step and the thermal profile protocol consisted of 45 annealing/extension cycles. Samples with lower C_t values contain relatively larger amounts of viral RNA. Negative samples were recorded as "No C_t", meaning that after 45 cycles the threshold level of fluorescence was

not reached. A sample of previously determined C_t -value of BTV RNA was used as a positive control, while BTV negative RNA was included as negative control. Each full 96 well PCR plate contained at least two replicates of negative and positive controls.

2.5.3 Gel electrophoresis (Agarose gel – AGE)

A 1% agarose gel was prepared by mixing 1g of electrophoresis grade agarose into 100 ml of 1x TAE buffer (Tris Acetate EDTA buffer, Sigma-Aldrich) in a conical flask. The flask was heated in the microwave until the agarose had dissolved completely (approximately 90 seconds). Agarose gel was then allowed to cool down until hand hot before adding approximately 5 µl of (10mg/ml) ethidium bromide per 100 ml of agarose solution. Gel was poured into the tray and an appropriate comb placed into the gel, to sweep any bubbles to the side of the tray and the gel allowed to set. After setting the comb was removed and 1xTAE poured to clearly cover the gel (approximately 700ml in a medium tank). On a piece of parafilm, using a pipette, 5µl of each cDNA (PCR product) sample was mixed with 2 µl of 10x loading dye/sample. Five µl of this mixed sample were loaded into an appropriate well in the gel. Five µl of working DNA 1Kbp ladder (Invitrogen) were also loaded into one or more wells in the gel, in order to indicate the size of any fragments. Samples were analysed using gel electrophoresis at 100V for 1.5 hours. Final products (DNA) were observed and photographed under UV light. The intensity depends on the concentration of the DNA. The size of the fragment was determined by comparison with the DNA bp ladder.

2.5.4 Sequencing of BTV strains

Primer design, amplification and sequencing of specific strains were performed in collaboration with Kyriaki Nomikou, a senior Postdoc member of the Molecular

Reference Research Group (AMRG) at IAH – Pirbright. Sequencing of purified PCR products was performed using the Sanger method with an Applied Biosystems 3730 BigDye ddNTP capillary sequencer (Maan *et al.*, 2010) or by using a 454 second generation sequencer (Caporale *et al.*, 2011) at the Welcome Trust Sanger Institute (UK), as part of a collaboration with IAH. Sequences were compiled, edited and the consensus sequences for all BTV segments from blood and passaged samples were analysed by using DNASTAR software (DNAStar Inc.) (Maan *et al.*, 2010).

Chapter 3: Optimisation and validation of quantification methods for bluetongue virus in adult *Culicoides*

Optimisation and validation of a high-throughput method for homogenization of *C. sonorensis* by Tissue Lyser instrument (qiagen, Crawley, UK) is presented. Infectivity and viral RNA amplification of *Culicoides* homogenates created using a Tissue Lyser and a traditional pestle and mortar technique are compared in both intrathoracically and orally infected *C. sonorensis*. These techniques were found to be comparable and an optimised programme for the Tissue Lyser was produced according to size and material of the beads used, the disruption programme (run length and frequency) and number of *Culicoides* processed.

3.1 Introduction

The detection of BTV in field-collected adult *Culicoides* represent an important tool for implicating species as vectors, as discussed previously in Chapter One. Previous studies of infection rates in *Culicoides* collected during epidemics of BTV in the northern Palaearctic and Mediterranean regions have relied upon either virus isolation in embryonated chicken's eggs (ECE) and BHK-21 cells (Caracappa *et al.*, 2003; Mellor & Pitzolis, 1979) or rtRT-PCR detection of viral RNA (Becker *et al.*, 2010; Ferrari *et al.*, 2005; Mehlhorn *et al.*, 2007; Vanbinst *et al.*, 2009). These methods have always been applied to homogenised pools of parous, unfed female *Culicoides*, collected using light-suction traps on farms where recent transmission of BTV occurred.

The use of pools of *Culicoides* allows rapid processing of large numbers of individuals and is useful in identifying the timing of virus transmission. Processing of

pools does, however, have the major disadvantage of being unable to distinguish *Culicoides* developing non-transmissible persistent infection from those possessing fully disseminated infections (Fu *et al.*, 1999; Jennings & Mellor, 1987). The proportion of adult *Culicoides* from European species that become infected in this way and remain unable to transmit the virus, is currently unknown, although a laboratory-based study using virus isolation recorded an infection rate varied between 7 13% among orally infected *C. pulicaris* s.l. and *C. obsoletus* s.l. (Carpenter *et al.*, 2006). As the infection rates that characterise even intense transmission of BTV in the field are usually low, this has commonly led to pools of *Culicoides* that most probably include only one infected individual being cited as evidence of vector status.

More recently a high-throughput Tissue Lyser / RT-PCR system was used to amplify RNA from individuals and pools of *C. sonorensis* that were either IT inoculated or orally infected with BTV (Kato & Mayer, 2007). Similar methods are in common use as part of other arboviral surveillance programs (Nasci *et al.*, 2002; Shi *et al.*, 2001). The Tissue Lyser / RT-PCR system developed, however, was not used to quantify BTV RNA in the samples concerned and hence could not be used to separate sub-transmissibly infected individuals. In addition, there was no assessment of whether the process of homogenisation itself reduced either infectious virus or BTV RNA load by physical disruption of samples.

It has previously been reported that individual adult *C. sonorensis* containing greater than 3 $\log_{10}$ $TCID_{50}$/individual, will possess a fully disseminated infection (Fu *et al.*, 1999). This chapter presents an optimisation of a Tissue Lyser-based method for homogenization of individual adult female *Culicoides*, suitable for both BTV isolation and quantification by virus titration or rtRT-PCR. The main advantage of the Tissue Lyser-based homogenization is the reduced processing time of samples, which is at least

24 times shorter comparing to the manual homogenization with mortar and pestle. The Tissue Lyser was compared to a standard grinding method (polypropylene motor-driven pestles), in terms of its sensitivity, reproducibility and speed of processing.

3.2 Material and method

All infection experiments were carried out using a Kosovo strain of BTV-9 (KOS2001/03) that had been passaged once through eggs and four times through BHK-21 cell culture (for further details on the virus see http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-9 and Table 2.1 in Chapter 2) due to the difficulty to work with field isolates of BTV. The infectivity of the initial virus preparation (tissue culture supernatant) was measured in BHK-21 cells as 6.75 $\log_{10}$ $TCID_{50}$/ ml (Chapter 2, Sections 2.1.1 and 2.4.1).

Groups of 2-3 day old colonised *C. sonorensis*, from the PIRB-s-3 strain (Boorman, 1974; Jones & Foster, 1969), were infected using semi-automatic intra-thoracic (IT) inoculation (Boorman & Mellor, 1975) (Chapter 2, Section 2.2.3) or by membrane-feeding (Chapter 2, Section 2.2.2). *Culicoides sonorensis* were IT inoculated using BTV-9 directly harvested from BHK-21 supernatant, while a 1:2 volume mixture of virus and horse blood was used to feed individuals through membranes (Mellor, 1971). Following IT inoculation / membrane feeding, *C. sonorensis* were either processed immediately or immobilized using CO_2 and the fully engorged female selected for incubation in pillboxes (Watkins & Doncaster, UK). A cotton pad moistened with a 5% granulated sugar solution was provided daily. After 10 days incubation at 25±1°C and 80% relative humidity (R.H.) surviving female *C. sonorensis* were selected for use.

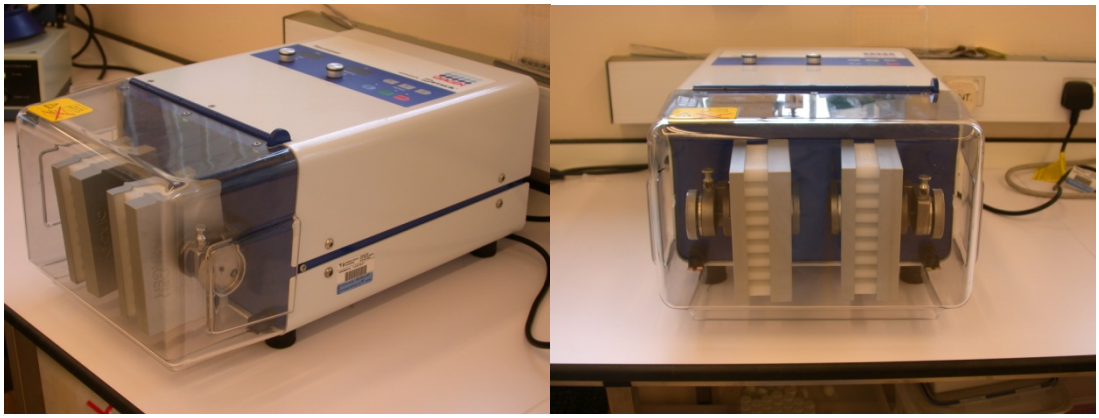

Figure 3.1: Tissue Lyser (Qiagen, Crawley, UK) instrument

3.2.1 Evaluation of possible reduction in the infectivity of BTV due to homogenisation treatment

The (Qiagen, Crawley, UK) (Figure 3.1) is capable of simultaneously homogenizing up to 96 samples, using high frequency shaking of each sample with or without a mechanical aid such as a ball bearing included with the sample. A preliminary test was carried out to investigate if stainless steel beads could potentially affect the infectivity of BTV-9 (KOS2001/03), during homogenisation of potentially infected adult *Culicoides*. Five different 1.5 ml Eppendorf tubes were filled with 1 ml of a ten-fold serial dilution of infected tissue culture supernatant (from 10^{-2} to 10^{-8}, diluted in Glasgow – MEM 'diluent') (Chapter 2, Section 2.1.1) and a stainless steel bead (1, 2, 3,4 and 5 mm diameter, one size for each tube). The tubes were shaken for 4 minutes at 30 Hz. The same sizes of beads and programme were also used to grind two non-infected *C. sonorensis* (1 midge/tube) as negative controls. A 1.5 ml Eppendorf tube, containing the same virus and using the same programme (but no bead) was used as a positive control. All of the samples were titrated in BHK-21 cells, with BTV-9 KOS2001/03 (diluted from 10^{-2} to 10^{-8}) as positive titration controls. . An increase on heating, possibly generated from the homogenization of the samples, was not recorded, but could potentially have had an impact on the infectivity of the virus.

3.2.2 The effect of Tissue Lyser disruption programme used on BTV detection

Initially seven disruption programmes involving varying duration and frequency of homogenization (based upon the manufacturer's recommendations) were tested, with 10 individual IT inoculated *C. sonorensis* allocated to each treatment group (see Table 3.1). These IT inoculated *C. sonorensis* were processed immediately following inoculation. Prior to homogenization, each adult insect was transferred individually into a 1.5 ml eppendorf tube containing 100 µl of Glasgow MEM 'diluent' (with 2 % antibiotics and 2% fungizone Chapter 2, Section 2.1.1). A standard 3 mm diameter stainless steel ball bearing (Dejay Distribution Ltd., Crowborough, UK) was then added to each tube and the contents homogenised for the allocated programme.

Following homogenisation, ball bearings were removed from each tube using a magnet, 900 µl of Glasgow MEM 'diluent' was added and the tubes were centrifuged at 13,000 X *g* for 5 min. Virus titrations of the supernatants were carried out on BHK-21 cell monolayers in 96-well microtiter plates (Chapter 2, Section 2.4.1). The monolayers were examined for cytopathic effects (CPE) at 3 and 5 days post-inoculation (Chapter 2 section 2.4.1). The BTV titre calculated from observation of CPE in titrated samples was expressed as $\log_{10}TCID_{50}$ / individual (Finney, 1964).

Presence of BTV was confirmed in all CPE positive samples using a rtRT-PCR assay (Chapter 2, section 2.5.2) (Shaw *et al.*, 2007) or enzyme immunoassay, ELISA (Chapter 2, section 2.4.1). To provide a comparison with earlier techniques, an additional 10 IT inoculated *C. sonorensis* were also ground individually in 1.5 ml eppendorf tubes using power-driven polypropylene pestles (Chapter 2 section 2.3). Assessment and calculation of BTV titre / individual was then carried out as for Tissue Lyser® processing.

3.2.3 The effect of ball bearing size and material used on BTV detection

The effect of ball bearing size and construction material were investigated using stainless steel balls measuring 1, 2, 3, 4 and 5 mm diameter and 3 mm polyethylene balls. In an initial experiment 10 IT-inoculated insects were homogenised individually with each ball stainless steel bearing size for 1 min at 25 Hz frequency (Table 3.2). In a second experiment 10 IT-inoculated individuals were homogenised individually using 3 mm stainless steel (only) or 3 mm polyethylene balls at a variety of frequencies (Table 3.1). BTV was detected and quantified through titration as described.

3.2.4 Validation of optimized technique and sensitivity with pooled samples

To validate the final homogenisation method, 40 IT-inoculated midges were individually homogenised with 2 mm stainless steel balls for 1 min at 25 Hz and compared with 40 insects ground individually via the power-driven pestle method. Additionally, two groups of 80 *C. sonorensis*, that had been orally fed on virus / blood suspension and incubated for 10 days (as previously described), were homogenized individually with the Tissue Lyser, or ground using the motorized pestle method, and their BTV titres assessed in an identical fashion.

Virus detection was also carried out using pools of midges with different virus positive: virus negative ratios (1:1, 1:5, 1:10, 1:25, 1:50 and 1:100). Positive *C. sonorensis* were produced via IT inoculation and incubated for 7 d at 25°C and 80% R.H. to allow full dissemination of BTV. Pools of insects were homogenised in a 1.5 ml Eppendorf tube, as previously described using the 2 mm stainless steel balls for 1 min at 25 Hz, and then 2 min at 30 Hz. Supernatants were titrated on BHK-21 cells (4 replicates/pool) (Chapter 2, Section 2.1.1 and 2.4.1). In all cases, AgELISA (Chapter 2,

Section 2.4.1) and rtRT-PCR (Shaw *et al.*, 2007) (see also Chapter 2, Section 2.5 for more details on this subject) assays were used to confirm the presence of the virus.

3.2.5 Statistical analysis

Following Levene's test for inequality of variance among the treatments used, the Kruskal-Wallace ANOVA followed by Tukey HSD was used to differentiate median titers among the eight treatments (all statistical testing performed using Minitab version 14: State College, PA). The effect of differences in stainless steel ball bearing size used on BTV detection was also analyzed by Kruskal-Wallace ANOVA. A Mann-Whitney U test was used to analyze differences among IT-inoculated midges (Section 3.2.4) homogenized with Tissue Lyser using 3 mm beads either in stainless steel of polyethylene *vs* power-driven.

3.3 Results

3.3.1 Evaluation of possible reduction in the infectivity of BTV due to homogenisation treatment

The preliminary results showed that shaking with stainless steel beads for 4 min at 30Hz reduced virus titres regardless of bead size. The virus titres of the 5 samples shaken with the stainless steel beads ranged from 5.25 to 5.5 $\log_{10}TCID_{50}$/ml, while the positive control was between 6.0 and 6.75 $\log_{10}TCID_{50}$/ml. The mean of the virus titres recorded in the tubes containing the stainless steel beads was of 5.4 $\log_{10}TCID_{50}$/ml, while the mean titre recorded in the positive control samples (without stainless steel bead) was 6.25 $\log_{10}TCID_{50}$/ml (Figure 3.2). All of the non-infected *C. sonorensis* samples gave negative results.

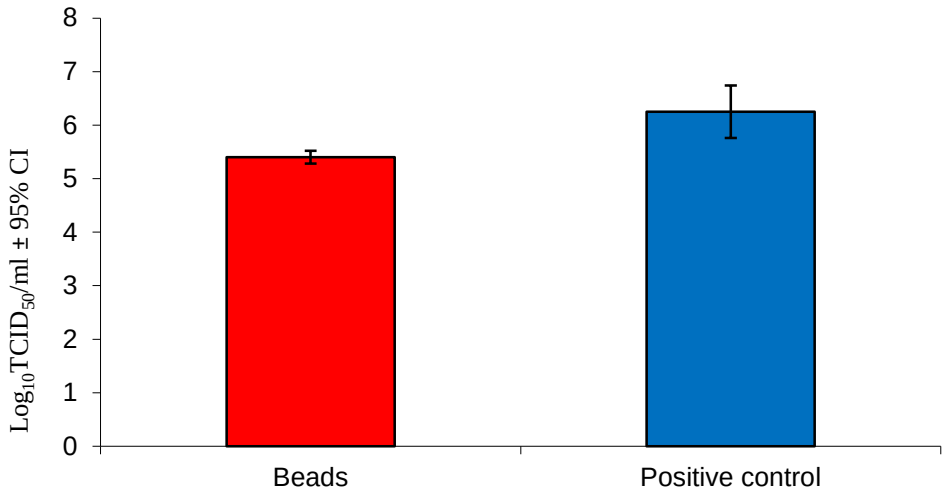

Figure 3.2: Mean titre recorded in the supernatant of BHK-21 cells infected with BTV-9 KOS after shaking in the Tissue Lyser using 5 different stainless steel beads'sizes (1, 2, 3, 4 and 5 mm diameter) for 4 min at 30Hz. The positive control was the same virus using same programme of the TL but without any stainless steal beads in the Eppendorf tubes.

3.3.2 The effect of Tissue Lyser disruption programme used on BTV detection

A significant difference was found in the quantity of infectious BTV detected following the use of different Tissue Lyser homogenisation programs (H = 57.73; df = 7; P <0.001), although no significant difference was noted in variance (W = 1.18; P = 0.325). Optimal homogenisation for survival of BTV was achieved by using relatively short runs in the Tissue Lyser (Table 3.1). Four of the programs used (1 min / 25 Hz; 30 sec / 20Hz : 30 sec / 30 Hz; 2 min / 25Hz; 1min / 25Hz : 1 min / 30Hz), showed no significant difference in the detected titre of BTV when compared with the standard power pestle method. The treatment of 1 min at 25 Hz gave the highest mean virus titre among the Tissue Lyser processed samples (2.58 ± 0.26 $\log_{10}$ $TCID_{50}$).

Table 3.1: Virus titre of intrathoracically inoculated *C. sonorensis* homogenised with 3mm stainless steel and polypropylene balls for different periods (minutes) and at different frequencies (Hz).

[a,b] Pairwise significant difference in groups recorded using Tukey HSD at $P < 0.05$.

[a,c] Pairwise significant difference in values recorded for each processing treatment using Mann-Whitney U at $P < 0.05$.

Treatment time / Frequency (Hz)	Mean virus titer (log_{10} $TCID_{50}$) ± 95% CI (n=10 for each treatment): Stainless steel balls	Mean virus titer (log_{10} $TCID_{50}$) ± 95% CI (n=10 for each treatment): Polypropylene balls
1 min / 25	2.32 – 2.84[a]	1.57 – 2.23[c]
30 sec / 20 : 30 sec / 30	2.28 – 2.68[a]	1.67 – 2.09[c]
2 min / 25	1.82 – 2.48[a]	1 – 1.7[c]
1 min / 25 : 1 min / 30	1.74 – 2.32[a]	1.07 – 1.57[c]
2 min / 25 : 1 min / 30	0.82 – 1.34[b]	-
2 min / 25 : 2 min / 30	0.69 – 1.25[b]	-
4 min / 30	0.12 – 0.94[b]	-
Power pestle	2.05 – 2.85[a]	

3.3.3 The effect of ball bearing size and material used on BTV detection

The use of different diameter stainless steel ball-bearings resulted in the recovery of significantly different quantities of virus ($H = 22.79$; df = 5; $P < 0.001$). Of the ball-bearing sizes tested, 1 and 5 mm diameter were significantly less effective than 2, 3 and 4 mm, which gave titres that did not differ significantly from those obtained using the pestle method (Table 3.2). Stainless steel balls (3mm) also performed significantly better than the polypropylene ones ($P < 0.01$ in all cases: Table 3.1).

Table 3.2: Virus titre of intrathoracically inoculated *C. sonorensis* homogenised using different diameter stainless steel balls for 1 minute at 25Hz frequency

[a,b] Pairwise significant difference in groups recorded using Tukey HSD at $P < 0.05$.

Ball bearing diameter (mm)	Mean virus titre (log_{10} $TCID_{50}$) ± 95% CI (n=10 for each treatment)
1	0.25 - 1.15[a]
2	1.64 – 2.06[b]
3	1.19 – 1.89[b]
4	1.07 – 1.77[b]
5	0.38 – 1.1[b]
Power pestle	1.21 – 1.89[a]

3.3.4 Validation of optimized technique and sensitivity with pooled samples

Individual homogenization of a further 40 IT-inoculated *C. sonorensis*, using an optimum combination of 2mm ball-bearings with a 1 min 25 Hz grinding program, gave titres that were not significantly different from those obtained for 40 individuals ground using the powered pestle method (mean ± 95% CI: Tissue Lyser = 1.48 ± 0.18; Power pestle = 1.60 ± 0.19: $W = 1526$; $P = 0.368$).

Thirty-five (44%) of 80 *C. sonorensis* that were infected by membrane-feeding and processed using the optimum Tissue Lyser assay contained detectable BTV at 10 days post-infection (Figure 3.3), of which twenty five (31%) contained greater than 3 $\log_{10}$ $TCID_{50}$ / individual. Forty eight (48%) of the 80 *C. sonorensis* homogenised using motorized pestles contained virus, of which twenty three (29%) contained greater than 3 $\log_{10}$ $TCID_{50}$ / individual.

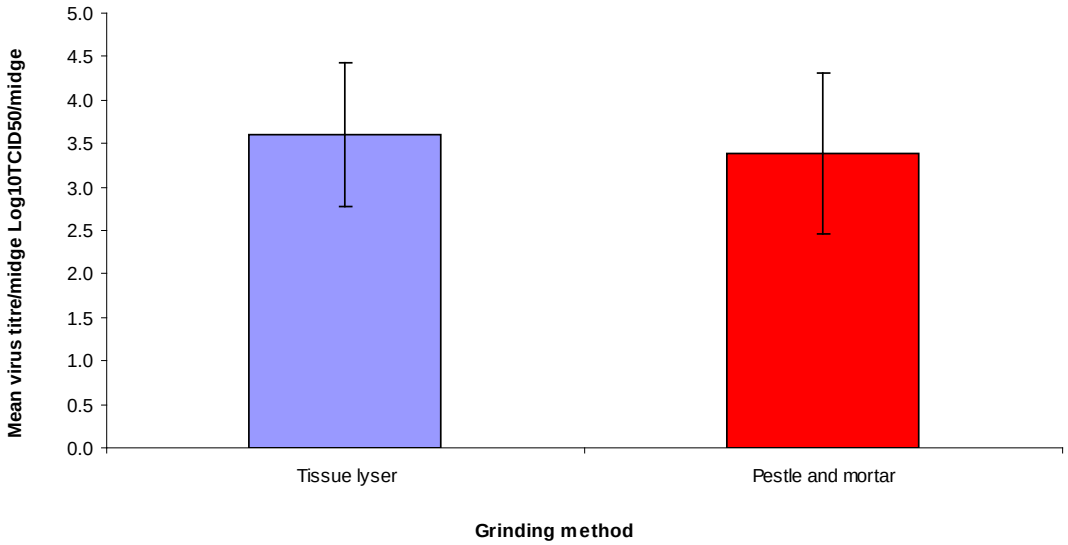

Figure 3.3: Mean virus titres in two groups of 80 *C. sonorensis* that had been orally fed with BTV-9 (KOS2001/03) then incubated for 10 days at 25 degrees.
The insects were homogenised individually, using either the Tissue Lyser (2 mm stainless steel beads for 1 min at 25Hz) or by mortar and pestle. Only the positive midges were included for calculation of the mean titres.

The maximum titre achieved in a single individual *C. sonorensis* using either Tissue Lyser or pestle method was 5 $\log_{10}$ $TCID_{50}$ / individual. No significant difference was found between the two methods in the mean titre of those midges containing ≥ 3 $\log_{10}$ $TCID_{50}$ / individual (W = 620.5; df = 44; P = 0.876) (Figure 3.3, 3.4). All midge homogenates that were positive for BTV by titration on BHK-21 cells also gave positive C_t values by rtRT-PCR. None of the sample that gave negative results after titration on BHK-21 cells were recorded as positive by the rtRT-PCR. A regression of C_t values against $\log_{10}TCID_{50}$/ml recorded in BHK-21 cells, shows a correlation (r^2) of 66% for the homogenates obtained with the mortar and pestle (Figure 3.4 a) and 48% for the Tissue Lyser (Figure 3.4 b). All the results obtained from the AgELISA matched the CPE recorded by the reading of the plates.

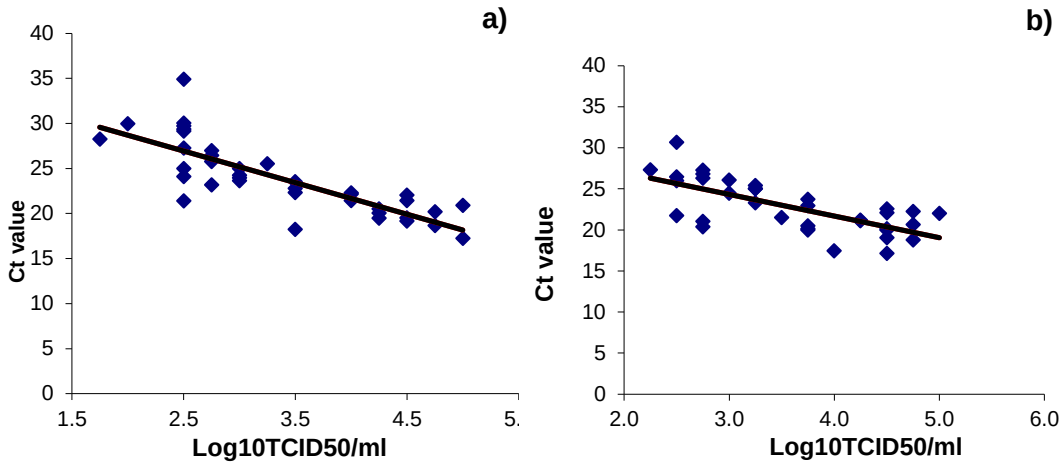

Figure 3.4: Plot of the quantities of virus detected by titration on BHK-21 cells and with rtRT-PCR recorded in *C. sonorensis* orally fed with BTV-9 (KOS2001/03) then incubated for 10 days at 25°C and homogenised with the Mortar and pestle method (a) y = -3.5138x + 35.73. R^2 = 0.6556 and **Tissue Lyser method (b)** y = -2.6297x + 32.206 R^2 = 0.4835.

3.3.5 Sensitivity of the TL method for detection of virus in pools of midges

Virus was detected in all pools of uninfected *C. sonorensis* containing single IT inoculated individuals with titres ranging from 4 to 6.75 $\log_{10}$ $TCID_{50}$ / pool (Table 3.3). All results were confirmed as positive by rtRT-PCR and indirect sandwich AgELISA.

Table 3.3: Infectious virus detected in pools of intrathoracically inoculated *C. sonorensis* (7 d.p.i.) processed using a Tissue Lyser after titration on BHK-21 cells ($\log_{10}TCID_{50}$/ml) and their C_t valued detected by rt-RTPCR of viral RNA.

***Culicoides* +ve': –ve ratio**	**Titre ($\log_{10}TCID_{50}$/ml)/C_t value**
1:1	5.5 / 20.54
1:5	6.0 / 21.16
1:10	6.0 / 20.41
1:25	≥6.5 / 21.49
1:50	≥6.5 / 21.5
1:100	6.0 / 21.12
1 (intrathoracically infected midges)	3.0 / 24.04
Positive control	6.75 / 17.76

3.4 Discussion

The aim of this study was to provide a standardised assay that could be used to quantify infectious BTV in individual *Culicoides* rapidly and accurately. It was envisaged that the development of this technique would allow the identification of vector(s) involved in outbreaks of BTV either in the laboratory using artificially infected *Culicoides* or through screening field collected individuals. While superficially similar to a previous study carried out using the Tissue Lyser system (Kato & Mayer, 2007), this study is the first to attempt to produce a high-throughput system that quantifies BTV prevalence in *C. sonorensis*. In addition, it is also the first to examine the potential for inactivation of BTV by physical disruption during processing of samples for vector competence studies. Further work is here suggested in order to reduce the gap between the two methods, for example by adding media that contains proteins (e.g. adult bovine serum) that may help in reduce the loss of infectivity.

The results of the study demonstrate that an optimal homogenisation programme of one minute disruption at 25 Hz using 2mm steel ball bearings was comparable to using a hand drive power pestle. This technique was successfully validated using IT inoculated *C. sonorensis* processed immediately following injection (which possess an inherently low variation in titre: (Fu *et al.*, 1999)) and membrane-fed individuals (that possessed a range of titres similar to those expected of infected *Culicoides* from the field: (Foster & Jones, 1979; Jones & Foster, 1978; Mellor, 1990). The titre of the recovered infectious BTV in both cases were similar to those obtained using the current standard technique of grinding with polypropylene pestles (Carpenter *et al.*, 2006; Nunamaker *et al.*, 2000; Savini *et al.*, 2004; Venter *et al.*, 2005; Walker & Davies, 1971). While the preparation time for homogenisation of individual samples is marginally increased by the time taken to add and remove the stainless steel balls from the Eppendorf tubes containing samples, the homogenization step itself is ~24 times faster in the current study (the Tissue Lyser can process either 48 or 192 samples simultaneously). This for the first time allows large-scale individual screening of both field and laboratory infected *Culicoides* for the presence of BTV, addressing the difficulties in interpretation highlighted for previous studies that screened pooled catches of individuals. The development of this technique also more easily enables the processing of large numbers of *Culicoides* for studies of variation in the extrinsic incubation period of BTV which is a major aim of the current thesis.

In a wider sense, the development of the current assay has considerable benefits for understanding *Culicoides*-BTV relationships worldwide. The straightforward standardisation in technology used reduces the probability of variation between users and laboratories in terms of processing (e.g. length of time and diligence spent processing each sample). The system will additionally be an economically viable one

for many laboratories in developing countries as the Tissue Lyser is a commonly used piece of equipment in reference laboratories for tissue disruption. Following the initial purchase of this equipment of approximately £3,000, running costs should also be minimal as the steel ball-bearings used in processing are substantially less costly than the polypropylene pestles used previously and do not have to be re-used, removing the possibility of sample contamination. The technique is also robust in allowing flexibility in processing programme and size of steel ball-bearing used.

A major challenge in promoting uptake of the assay by users lies in the fact that processing of pools of *Culicoides* is even more rapid than the technique used here. Hence, emphasising the importance of sub-transmissible infections in confounding vector-implication is vital, but this is regularly ignored in studies utilising pool-processing (Becker *et al.*, 2010; Vanbinst *et al.*, 2009). A second important factor is that the production of individual homogenates allows retrospective assigning of species supporting BTV replication using molecular markers in a secondary assay (Gomulski *et al.*, 2005; Mathieu *et al.*, 2007; Pages & Monteys, 2005). This has considerable importance where species cannot be straightforwardly separated by their morphology (a common scenario in global *Culicoides*-BTV systems: (Casati *et al.*, 2009; Meiswinkel *et al.*, 2004a)). A final consideration is the ability to adapt the current system to current diagnostic technology available in BTV reference laboratories worldwide. Increasingly, most laboratories utilise rtRT-PCR as a frontline technique which is more rapid and have the potential for a much higher throughput substantially increase the speed of processing (Shaw *et al.*, 2007) The detection of infectious BTV using cell-based isolation in the current study indicates that the system developed is unlikely to impact upon the quantity of BTV RNA detected (Mertens *et al.*, 2009).

The relationships that exist between threshold cycle (C_t) values generated by rtRT-PCR, virus titre and transmissibility of infection still need to be established and are addressed in Chapters 4 and 5. If comparable, this may enable virus isolation/titration to be replaced with rtRT-PCR assays. . Pairing of the technique with conventional or rtRT-PCR assays for *Culicoides* species identification (e.g. for the north-western Palaearctic species) (Cetre-Sossah *et al.,* 2008; Gomulski *et al.,* 2006; Nolan *et al.*, 2007; Pages & Monteys, 2005; Stephan *et al.,* 2009) should prove to be straightforward, given the sensitivity already achieved with these methods and the fact that identification of DNA from different insect species would not require quantification. Due to difficulties in successfully feeding northern Palaearctic species of *Culicoides,* this study used adults of *C. sonorensis* as a model species of vector. Chapters Four, Five and Six of this thesis will additionally apply this technique to other *Culicoides* species and demonstrate that the system can be applied to these putative vectors. In conclusion, a new high throughput method for rapid detection of BTV infection in *Culicoides sonorensis* by using Tissue Lyser instrument (Qiagen, Crawley UK) has been validated. The data presented indicate that a disruption programme of 1 minute at 25Hz with a stainless steel bead of 2 mm did not show any stastistical significant reduction in titre when compared to the classical standard homogenization process with mortar and pestle. Infectivity was still detectable in a pool of 100 intrathoracic inoculated midges with a [+ve: -ve] infection rate of 1:100.

Chapter 4: Optimization of techniques to determine BTV infection rate and dissemination in *Culicoides*

The use of insect cells (KC *C. sonorensis*) for investigation of BTV dissemination in both orally and intrathoracically infected *C. sonorensis* is investigated. In order to confirm virus amplification in this KC cell based assay, which does not show cytopathic effect, a validation of an AgELISA was carried out. Correlation between virus infectivity ($log_{10}TCID_{50}$/ml inferred by AgELISA) and viral RNA load (as measured by C_t values using real time RT-PCR) in dissected or whole body artificially infected *C. sonorensis* are assessed. This demonstrates that the KC *C. sonorensis* cell line can be used as a sensitive detection assay system for infectious virus in *Culicoides*.

4.1 Introduction

Early research on BTV relied upon the detection of clinical signs in hosts that exhibited clinical disease to define the presence or absence of infectious virus (Erasmus, 1975; Foster *et al.*, 1963; Hardy & Price, 1952; Price & Hardy, 1954; Spreull, 1905). The discovery that BTV caused lethal hemorrhaging in embryonated hen's eggs was a significant advance in the study of BTV, not only providing a means of repeatedly passaging the virus, but also a detection system, a means of testing pathogenicity of strains and a route for the manufacture of live-attenuated vaccines (Alexander 1947; Foster and Luedke 1968). Following this discovery, several cell cultures were also shown to support BTV replication, including Baby Hamster Kidney-21 cells (BHK-21), African green monkey kidney cells (VERO) (Bando, 1975) and calf pulmonary artery

endothelial bovine cells (CPAE) (Wechsler & Luedke, 1991; Wechsler & McHolland, 1988). Subsequently, the systematic sampling of BTV strains was commonly conducted by reference laboratories, through passage of field strains into embryonated hen's eggs, followed by passage in BHK-21 cells (Ballinger *et al.*, 1987; Kirkland & Hawkes, 2004). This not only formed a convenient experimental framework, in comparison to previous studies that were reliant upon direct animal infection, but also supported the production of improved live-attenuated vaccines by plaque selection (Roy *et al.*, 2009).

In studies of vector competence, the adoption of more convenient laboratory model-systems has facilitated a wide range of studies (as discussed in Chapter 1). There was concern, however, that the adaptation of arboviruses to specific cell lines would result in selection of specific and possibly non-representative sub-populations, with differences in virulence characteristics (Weaver, 2006). For this reason, there was interest in the use of insect cells lines as an alternative means of propagating and detecting BTV. This led to the production of cell line derived from embryos of *C. sonorensis* which were found to support BTV replication, albeit without any observed cytopathic effect (CPE) (Wechsler & Luedke, 1991; Wechsler & McHolland, 1988). The KC cell line subsequently produced was highly sensitive to BTV strains (Wechsler *et al.*, 1989). Despite the lack of CPE, infection of KC cells can be confirmed by testing the cell-culture supernatant using AgELISA, or by infection of a susceptible cell line that shows CPE following virus replication (e.g. CPAE or BHK-21).

A second major advance was the development of diagnostic assays for BTV based on reverse transcriptase polymerase chain reactions (RT-PCR). Initially these PCR assays used a 'conventional' format, generating cDNA amplicons that could be analysed initially by 1%AGE then confirmed by sequence analyses (Maan *et al.*, 2007a; Mertens *et al.*, 2007). By targeting Seg-2, which controls the virus serotype, these

assays can be used to detect and identify the different BTV serotypes (Maan *et al.*, 2012a; Maan *et al.*, 2008). Together with advances in genome sequencing of BTV strains and associated phylogenetic analyses, these techniques have revolutionised detection of BTV in the field having the advantage of being substantially more rapid, sensitive and specific than the virus-isolation-based and serological techniques they have largely replaced. In addition, the more recent use of real-time RT-PCR (rtRT-PCR), has provided a more sensitive detection of viral RNA, in a closed tube format that helps avoid cross contamination and therefore false positive results.

Following recent incursions of BTV into northern Europe, a series of studies were conducted to explore the role in transmission of adult *Culicoides* collected at outbreak sites (Dijkstra *et al.*, 2008; Mehlhorn *et al.*, 2007; Meiswinkel *et al.*, 2007). These studies utilized rtRT-PCR to detect viral RNA within pools of *Culicoides* (without subsequent virus isolation) largely because this technique was readily available in national reference laboratories for BTV that were already processing large numbers of ruminant-derived samples. A lack, however, of an estimated BTV-RNA quantification means that results were difficult to interpret, as 'positive' findings may represent inactivated BTV that had persisted in the *Culicoides* following an infected blood meal, or sub-transmissible infections, which commonly occur in this genus, as reviewed in Chapter one (Mellor, 2000). Since these early studies, the inclusion of cycle threshold (C_t) values as a proxy indication of RNA quantity has become more common (Hoffmann *et al.*, 2009b; Hoffmann *et al.*, 2009c; Vanbinst *et al.*, 2009), although without a clear threshold value and consistent methodologies, this does not directly distinguish sub-transmissible infections from fully disseminated ones.

This chapter compares the sensitivity of an rtRT-PCR based assay to that of virus isolation and quantification by titration on the KC cell line. This will make it

possible to use these RNA based techniques to detect persistent infection of *Culicoides* cells with BTV, even though they do not show cytopathic effects (CPE). An AgELISA was also evaluated for the detection of BTV infection of insect cell cultures, avoiding the requirement for adaptation of the virus and titration-assay transfers to mammalian cell cultures (which do show CPE).

Virus titre of two strains of BTV possessing different passage history (egg and BHK-21 cell or KC cell adapted) was evaluated by observation of CPE after replication on BHK-21 cells. Virus titration of same strains was additionally carried out on KC cells. Due to the lack of cytopathic effect in KC cells, an Antigen Elisa assay, enabling detection of virus antigens/particles in the supernatant of KC cells, was used to assess infectious titre. Evaluation of the specificity and sensitivity of this assay was also described. Correlation of infectivity ($log_{10}TCID_{50}$/ml) obtained by AgELISA and viral RNA load (Ct value) using real time RT-PCR, was carried out on BTV artificially infected *Culicoides* (by intrathoracic inoculation and by oral feeding).

4.2 Material and Methods

4.2.1 Validation of methods to detect BTV replication in KC cells

Two strains of BTV, previously adapted in either BHK-21 or KC *C. sonorensis* cells, were used in this experiment (Chapter 2, Section 2.1.1, 2.1.2):

1) BTV-9 KOS2001/02 E_1BHK_6, derived from BTV-9 KOS2001/02 E_1BHK_1
2) BTV-8 KC_5 derived from the BTV-8 UKG2007/06 sheep blood.

A ten-fold serial dilution series of each of the two virus strains was titrated on 96 well plates, containing monolayers of BHK-21 cells and/or KC cells. Plates were incubated

for 7 days at 37°C with 5% CO_2 (BHK-21 cells) or at 25°C (KC cells) and examined (BHK-21 cells) for cytopathic effect (CPE) at days 3, 5 and 7 (Chapter 2, Section 2.4.1). Since KC cells do not develop CPE, they incubated for 7 days, then 100µl/well of supernatant from the potentially infected KC cell plate, was transferred into a new 96 well plate containing monolayers of BHK-21, sealed and incubated for a further 7 days at 37°C with 5% CO_2 (see scheme in Chapter 2 Figure 2.5). These BHK-21 cell plates were examined for CPE at days 3, 5 and 7 post-inoculation (Figure 4.1)..

Supernatant aliquots from infected KC and BHK-21 cells were also tested in an Ag ELISA. This assay (Figure 4.2) is a modification of a previous antigen sandwich ELISA developed for AHSV (Hamblin *et al.*, 1991; Thevasagayam *et al.*, 1996). Each well of a 96 well immunoplate was coated with 50µl/well of primary polyclonal rabbit antibody 'Orab277' (anti purified BTV-1 ref.strain RSArrrr/01 diluted in coating buffer 1:2000) and incubated overnight at +4ºC. Following incubation, the immunoplates were washed 3 times in non-sterile PBS and dried by inversion on absorbent paper toweling. Supernatant from plates containing KC or BHK-21 cells that had previously been infected with BTV, was then applied to each well (50µl/well). After 45-60 minutes incubation at 37°C, the plates were washed with PBS as described above and coated with a secondary polyclonal guinea-pig antibody 'Orab279' (anti purified BTV-1 ref.strain RSArrrr/01 diluted 1:2000 into blocking buffer: non-sterile PBS supplemented with 0.1% Tween-20 and 5% Marvel milk). Polyclonal rabbit anti-guinea pig immunoglobulins/HPR (DAKO, UK Ltd.) (1:1000 dilution in 5% marvel blocking buffer) were then added and plates incubated for further 45 minutes at 37°C. Each plate was then washed three times with PBS and 50 µl/well of substrate was added (0.4 mg/ml o-Phenylenediamine dihydrochloride (Sigma, UK) and 0.05% hydrogen peroxidase solution). Plates were then left at room temperature for 10 minutes.

Following this, 50µl of 1M sulphuric acid was used to halt the reaction and plates read with a spectrophotometer at a wavelength of 492nm using a cut off of <0.20 optical density. Infectious supernatant from the BHK-21 cells titrated with same two BTV strains, were also tested by AgELISA test, in order to compare differences in the virus titre between the two assays.

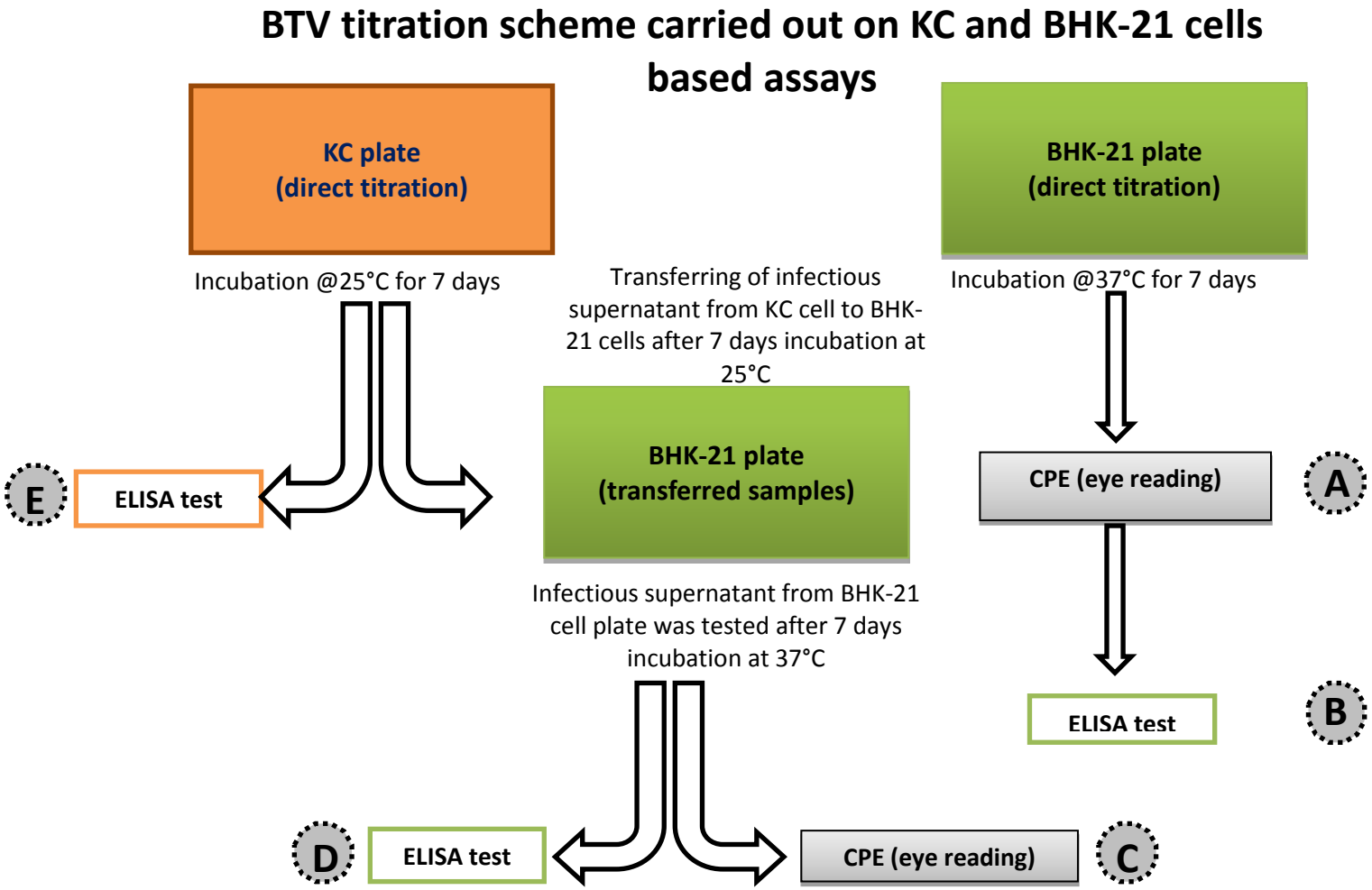

Figure 4.1: BTV titration scheme carried out on KC and BHK-21 cells based assays.
Results obtained from each test (highlighted here with different letter symbols A-E) are reported in Table 4.1 of this Chapter.

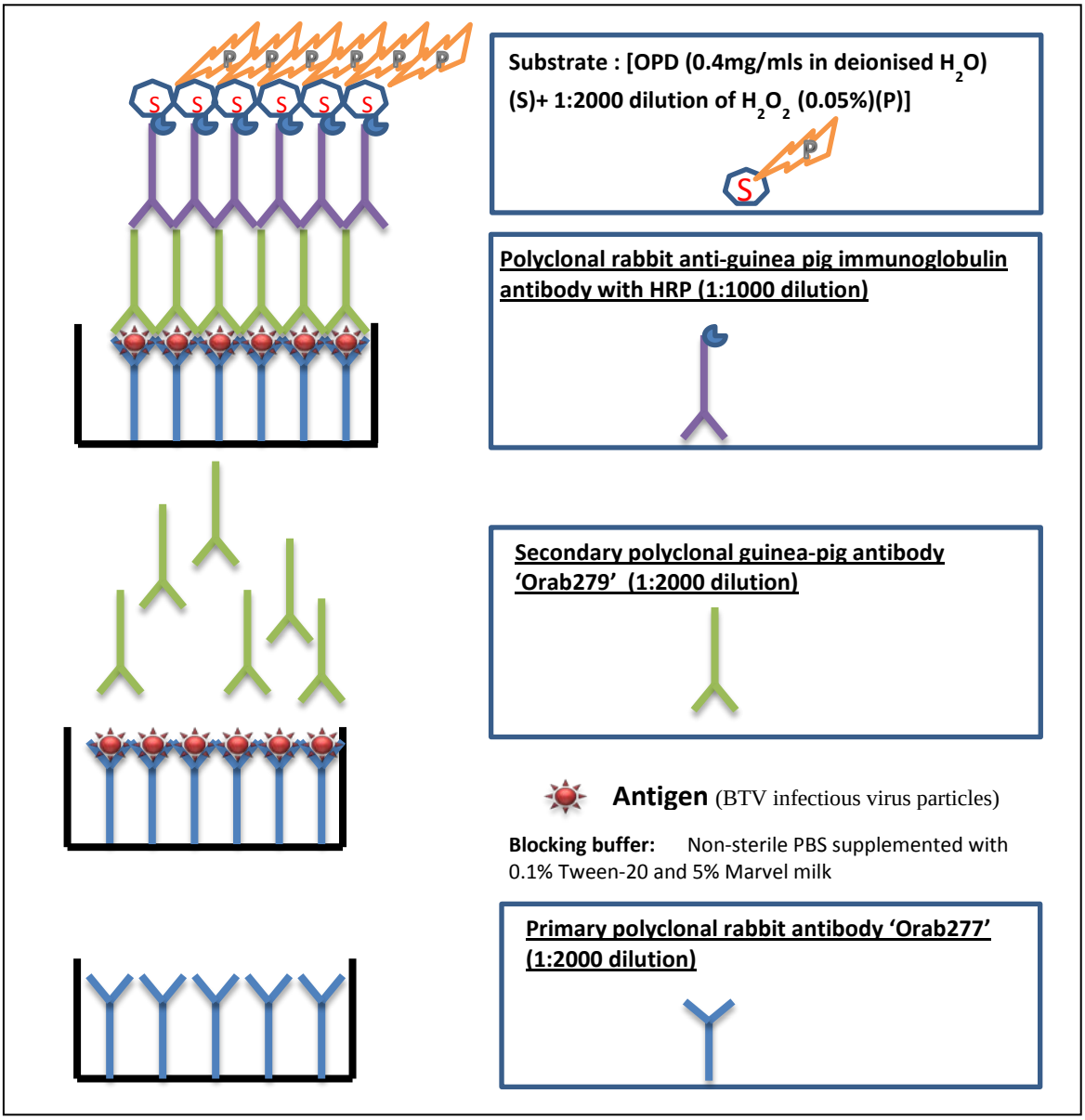

Figure 4.2: Description of AgELISA stages.
Immunoplates were coated overnight (+4°C) with primary polyclonal rabbit antibody 'Orab277' (anti purified BTV-1 ref.strain RSArrrr/01) (50µl/well) diluted in coating buffer (1:2000). After incubation, plates were washed 3X in non-sterile PBS followed by coating with antigen (infectious BTV supernatant) (50µl/well). After 45-60'incubation @+37°C plates were washed as above and coated with a secondary polyclonal guinea-pig antibody 'Orab279' (anti purified BTV-1 ref.strain RSArrrr/01) diluted 1:2000 into blocking buffer (non-sterile PBS supplemented with 0.1% Tween-20 and 5% Marvel milk). After incubation and washing as above, polyclonal rabbit anti-guinea pig immunoglobulins/HPR (DAKO, UK Ltd.) (1:1000 dilution in 5% marvel blocking buffer) were added and plates incubated for 45'@37°C. After final wash with PBS (X3), 50 µl/well of substrate was added (0.4 mg/ml o-Phenylenediamine dihydrochloride (Sigma, UK) and 0.05% hydrogen peroxidase solution). Plates were left at room temperature for 10 minutes. Following this, 50µl of 1M sulphuric acid were added to each well to halt the reaction and plates were read with a spectrophotometer at a wavelength of 492 nm using a cut off of <0.20 optical density.

4.2.2 Characterization of a cut-off values based on the specificity and sensitivity of the test

Optical density reading (OD) values were analyzed using a linear mixed model by Dr S. Gubbins Senior Postdoc scientist in the mathematical modeling research group at Pirbright (IAH, UK), to investigate whether any significant difference existed between the OD values for KC cells and homogenized midges. The model included Log OD as a response variable and, whether the sample tested was originated from KC cells or *Culicoides* homogenates, as a fixed effect and plate as a random effect. A second analysis on the sensitivity and specificity of the AgELISA for different cut-offs and the Receiver-Operator Characteristic (ROC) curve for the ELISA, which assesses the discriminatory power of the ELISA, was also carried out. For the test, the following samples were used: 1) negative supernatant of KC cells; 2) negative homogenates of *C. sonorensis*, and 3) supernatant from KC cells infected with a ten-fold serial dilution of both BTV-8 UK2007/06 KC_5 and BTV-9 KOS2001/02 E_1BHK_6 described in section 4.2.1. Statistical analysis was done using R (Team, 2012).

4.2.3 Examining the level of BTV dissemination in adult Culicoides *by virus titration on KC cells and real time RT-PCR*

Adults of two different *Culicoides* vector species were used in these experiments: *C. sonorensis*, a North American vector of BTV (Foster *et al.*, 1963; Price & Hardy, 1954) and *C. imicola*, a South African vector for BTV and African horse sickness virus (Du Toit, 1944; Mellor, 1990; Walker & Davies, 1971). Adults of these *Culicoides* species were infected with BTV-1 GIB2007/01 KC_2 (C_t = 14.88, 7.0 $\log_{10}TCID_{50}$/ml) using two different techniques: by intrathoracic inoculation (Chapter 2, Section 2.2.3) and by oral feeding with a mixture of blood and infected cell-culture supernatant (Chapter 2, Section 2.2.1 and 2.2.2). As a negative control, 6 uninfected *C. sonorensis* were also

analysed in parallel with the infected insects. Dissemination of BTV in *Culicoides* was assessed by virus detection in each part of the insects' body, using both rtRT-PCR (with duplicate extractions run on the same plate), and titration of a ten-fold serial dilution on KC-cells, followed by AgELISA (Section 4.2.1).

4.2.3.1 Intrathoracic Inoculation (IT) of adult Culicoides

Approximately 100 *C. sonorensis* were inoculated with BTV-1 GIB2007/01 KC_2 using the method described in Chapter 2, Section 2.2.3. Following inoculation, surviving *C. sonorensis* were transferred to pill boxes and incubated for 11 days at 25°C. Saliva, head and abdomen/thorax were separately collected from each individual and stored at +4°C (Chapter 2, Sections 2.3.1, 2.3.2).

4.2.3.2 Oral Infection of adult Culicoides

Culicoides sonorensis and *C. imicola* were fed through an artificial parafilm membrane or through a chicken skin membrane, on a blood mixture of defibrinated horse blood and BTV-1 GIB2007/01 KC_2 (1:2 dilution) which had a titre of 7.0 $\log_{10}TCID_{50}$/ml and viral RNA equal to 14.88 C_t (Chapter 2, section 2.2.1 and 2.2.2). After 7 days incubation at 25°C, 60 surviving individuals were selected under light using CO_2 anaesthesia (*C. sonorensis*), or on a chill table (*C. imicola*). For each individual, saliva, head and abdomen/thorax were separately collected and homogenized (Chapter 2, Sections 2.3.1, 2.3.2) and then stored at +4°C.

4.2.4 Viral detection in orally infected adult C. sonorensis *and* C. nubeculosus

Oral infection of *C. sonorensis* and *C. nubeculosus* was carried out using membrane-based methods (see Chapter 2, Section 2.2.1) and a BTV-1 GIB2007/01 KC_3

strain (same described in 4.2.3.2 but passaged 3 times on KC cells) mixed with defibrinated horse blood and used at a recorded titre 7.5 $log_{10}TCID_{50}$/ml and a C_t value of 16.74. Several hundred colony derived individuals of each species were fed and selected under light CO_2 anaesthesia. At 24 hour time intervals following feeding, 25 surviving individuals of each species were removed and processed using the Tissue Lyser method (Chapter 3) and BTV RNA quantified using the rtRT-PCR assay (Chapter 2, Section 2.5.2) and the titre of infectious BTV assessed by titration on the KC cells followed by AgELISA (Section 4.2.1). Virus titres and C_t values were compared as a time series dataset and between the two species and dissemination defined from the results of 4.2.3.

4.2.5 Investigation of BTV dissemination *in* C. sonorensis *by confocal microscopy*

A preliminary study on localisation of virus dissemination in infected *C. sonorensis*, by confocal microscopy, was also carried out. Two-three days old *C. sonorensis* were injected with BTV-1 (GIB2007/01 KC_2). Longitudinal sections were taken of the *C. sonorensis* (Chapter 2, Section 2.3.3) and immunolabelled with polyclonal NS2 rabbit antibodies ('Orab1'NS2 – IAH), followed by a secondary species-specific goat-anti-rabbit IgG, conjugated to fluorescence Alexa Fluor® 488 (green) (Chapter 2, Section 2.3.4).

4.3 Results

4.3.1 Validation of an antigen ELISA, to detect virus antigens / particles in the supernatant of KC cells after titration / infection with BTV

BTV replication data obtained by titration of BTV strains previously adapted to BHK-21 or KC cells is presented in table 4.1. The BTV-8 strain UKG 2007/06 produced limited CPE in BHK-21 cells, although greater quantities of BTV were detected using the

AgELISA. Passage of this virus strain on BHK-21 cells did not substantially improve the agreement between CPE observed and subsequent AgELISA. In contrast, with the BTV-9 KOSOVO E_1BHK_6 strain, CPE was detected across the BHK-21 cell line experiments and agreed closely with results of the AgELISA.

Table 4.1: Infectivity ($log_{10}TCID_{50}$/ml) of BTV-8 UKG2007/06 and BTV-9 KOS2001/02 E_1BHK_6 inferred by observation of cytopathic effect (CPE) in BHK-21 cells or measured by AgELISA after titration on KC and BHK-21 cells (as indicated).

Virus detection methods	**BTV-8 UKG 2007/06 (KC_5)** ($log_{10}TCID_{50}$/ml)	**BTV-9 KOS2001/02 (E_1BHK_6)** ($log_{10}TCID_{50}$/ml)
BTV strains titrated directly on BHK-21 cells	2.25 / 6.5 (CPE detection / AgELISA)	6.0 / 4.5 (CPE detection / AgELISA)
BTV strains titrated on KC cells and then transferred on a BHK-21 cells plate	3.0 / 6.5 (CPE detection / AgELISA)	5.6 / 5.6 (CPE detection / AgELISA)
BTV strain titrated on KC cells and detected using AgELISA	6.75 (AgELISA)	6.0 (AgELISA)

4.3.2 Evaluation of the specificity and sensitivity of the AgELISA

Analysis of the data showed no significant difference between the OD values obtained in the AgELISA for negative KC cells supernatant and homogenised uninfected midges (P=0.92) and that plate-to-plate variability accounts for about 11% of the variation as a whole. Data from analyses of the sensitivity and specificity of the ELISA (Figure 4.3) for different cut-off values and the Receiver-Operator Characteristic (ROC) curve for the ELISA, suggest that setting a cut-off of around 0.16-0.18 provided a high specificity (>95%), without significant loss in sensitivity.

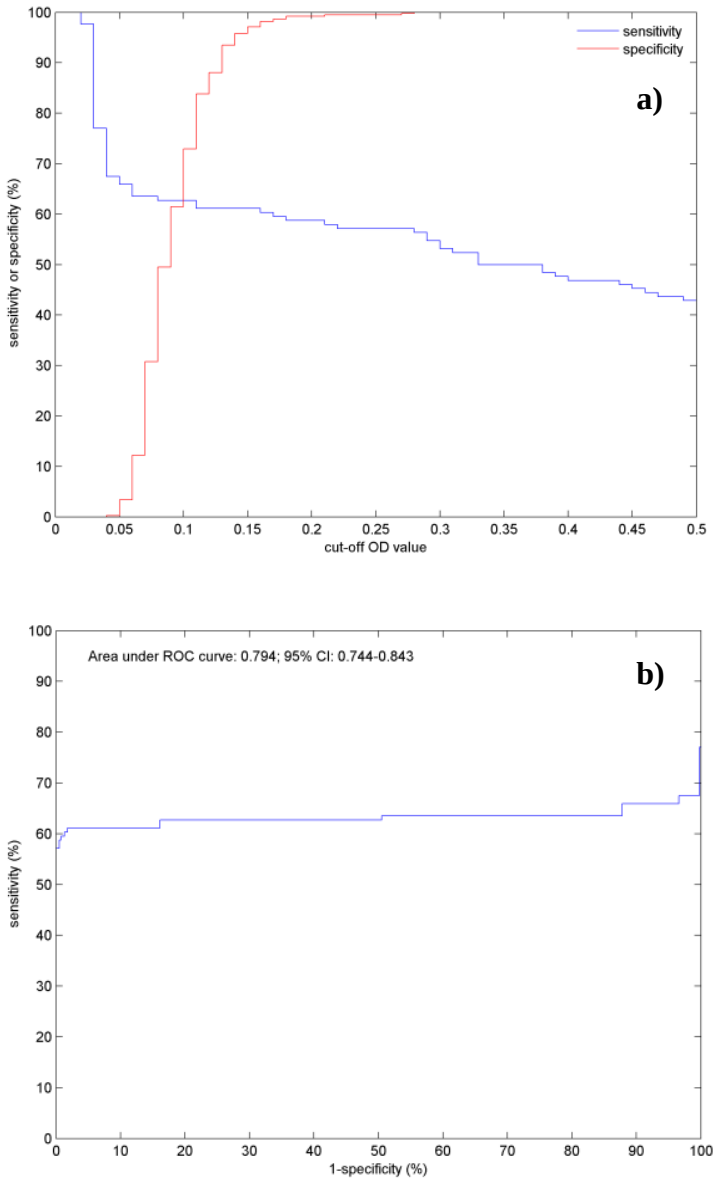

Figure 4.3: a) Analysis of the sensitivity and specificity of the AgELISA. b) Receiver-Operator Characteristic (ROC) curve.

4.3.3 Examining the level of BTV dissemination in Culicoides *by virus titration on KC cells and real-time RT-PCR*

4.3.3.1 Dissemination of BTV in intrathoracically inoculated C. sonorensis

The C_t values obtained by rtRT-PCR assays of uninfected *C. sonorensis*, were either above the standard assay cut-off of 40 C_t, or did not produce a C_t value. Survival rates of

C. sonorensis intrathoracically inoculated with BTV-1 GIB2007/01 KC_2 were low, with only approximately 6% of individuals surviving the 11 day incubation period. All six surviving individuals possessed fully disseminated infections that could be detected by rtRT-PCR assay and by virus isolation (Table 4.2). The largest quantity of viral RNA was found in the thorax and abdomen (Mean C_t = 18.32 ± 0.52 95% CI) followed by the head (Mean C_t = 22.73 ± 1.60 95% CI). C_t values recorded from the saliva collected ranged from C_t's of 27.8 to 32.14, while excised salivary glands from one female produced a significantly higher amount of viral RNA with higher C_t (21.16-21.43) when compared to the results obtained from the saliva in the other individuals which ranged between 27.8-32.14 C_t value (Table 4.2).

Table 4.2: Virus titres in different body parts of *C. sonorensis* injected with BTV-1 GIB2007/01 KC_2 (C_t:14.88 Titre: 7.0 $log_{10}TCID_{50}$/ml). BTV RNA (C_t) and infectious virus titre ($log_{10}TCID_{50}$/ml) were measured in duplicate homogenates of different part of the body of *C. sonorensis* intrathoracically injected with this strain. *Salivary glands excised

Female	Abdomen and Thorax		Head		Saliva	
	RT-PCR (C_t)	Titre ($log_{10}TCID_{50}$ / ml)	RT-PCR (C_t)	Titre ($log_{10}TCID_{50}$ /ml)	RT-PCR (C_t)	Titre ($log_{10}TCID_{50}$/ml)
1	18.23/17.71	≥5.5	22.22/22.30	3.5	31.09/31.99	2.75
2	17.75/17.74	5.25	22.2/21.96	3.75	no Ct/32.14	2.25
3	18.89/18.71	≥5.5	20.86/21.10	4.75	27.8/28.05	3.75
4	19.14/18.49	≥5.5	22.14/22.43	4.5	28.66/28.99	2.5
5	17.45/17.54	≥5.5	22.30/22.42	5	30.3/30.30	2.75
6*	18.48/18.72	≥5.5	26.66/27.00	3	21.16/21.43	4.75

Levels of infectious virus were broadly consistent with total BTV RNA, with homogenates of abdomen/thorax ranging between 5.25 and ≥5.5 $log_{10}TCID_{50}$ / ml, heads containing 3 to 5 $log_{10}TCID_{50}$/ ml and saliva 2.5-3.75 $log_{10}TCID_{50}$/ ml.

4.3.3.2 Dissemination of BTV in orally infected C. sonorensis *and* C. imicola

Using membrane feeding, 19 of 60 *C. sonorensis* (32%) that were infected with BTV-1 GIB2007/01 KC_2 (Titre: 7.0 $log_{10}TCID_{50}$/ml C_t:14.88) contained BTV RNA (testing of samples in duplicate at day 7 post-infection). Nine of these *C. sonorensis* (15%) contained virus in the head and thorax/abdomen, indicating a fully disseminated infection (Table 4.3). Nine of the individuals processed possessed C_t values indicative of partially disseminated infection with viral RNA detected in the thorax/abdomen or abdomen only. Forty two flies (70%) either did not give a C_t value or possessed traces of BTV RNA in a single body part only, which was not indicative of active replication. BTV RNA was also detected in the saliva of two of the fully disseminated individuals, albeit at high C_t values (33.47 and 36.22).

Table 4.3: Levels of dissemination of BTV in *Culicoides sonorensis* membrane fed with BTV-1 GIB2007/01 KC_2 (Titre: 7.0 $log_{10}TCID_{50}$/ml C_t:14.88) inferred from rtRT-PCR assays : BTV RNA loads (C_t) (n=60).

Level of dissemination in *C. sonorensis*	n	**Abdomen** Mean C_t (± 95% CI)	**Thorax** Mean C_t (± 95% CI)	**Head** Mean C_t (± 95% CI)
No detectable/trace RNA	42	No Ct	No Ct	No Ct
RNA abdomen only	5	31.6 – 35.2	No Ct	No Ct
RNA thorax/abdomen	4	27.4 – 34.2	30.03 – 35.9	No Ct
RNA head, thorax/abdomen	9	22.98 –28.98	22.4 – 27.2	25.2 – 28.8

Infectious BTV was isolated from the abdomens and thorax of all fully disseminated individuals except one that yielded low C_t values from all body parts (Abdomen = 35.84; Thorax = 33.39) (Table 4.4). Successful isolations were also made from seven of the nine heads (failing sample C_t's = 32.79 and 28.55 – the lowest values recorded within the cohort). The success of BTV isolation from partially disseminated

individuals was far more variable, ranging from 50% in abdomens and thorax infections (2/4 samples in both cases) to 16.6% for those with BTV RNA detected in the abdomen only (1/6).

Table 4.4: Levels of dissemination of infectious BTV recorded in *Culicoides sonorensis* membrane fed with BTV-1 GIB2007/01 KC_2 (Titre: 7.0 $log_{10}TCID_{50}$/ml C_t:14.88) inferred from virus titration on KC cells followed by AgELISA

Level of dissemination in *C. sonorensis*	N	**Abdomen** Median (range) Titre ($log_{10}TCID_{50}$/ml)	**Thorax** Median (range) Titre ($log_{10}TCID_{50}$/ml)	**Head** Median (range) Titre ($log_{10}TCID_{50}$/ml)
No detectable/trace RNA	42	No infectivity detected	No infectivity detected	No infectivity detected
RNA abdomen only	5	1.5 (≤0.5-1.5)	No Ct	No infectivity detected
RNA thorax/abdomen	4	2.88 (≤0.5-3.5)	1.25 (≤0.5 – 1.75)	No Ct
RNA head, thorax/abdomen	9	3.5 (≤0.5 - ≥6)	3.4 (≤0.5 - 4)	1.5 (≤0.5 - 2.25)

The range of viral RNA levels detected among the different body parts of the orally infected *C. imicola* (Table 4.5) was similar to that of *C. sonorensis*, although the mean RNA in each body part and for each level of dissemination was slightly lower in all cases. BTV RNA was detected in the saliva of 8 of the fully disseminated individuals (C_t values ranged from 35.12-30.77).

Table 4.5: Levels of dissemination of BTV in *C. imicola* membrane fed with BTV-1 GIB2007/01 KC_2 (Titre: 7.0 $log_{10}TCID_{50}$/ml C_t:14.88) inferred from rtRT-PCR assays : BTV RNA loads (C_t) (n=60).

Level of dissemination in *C. imicola*	N	**Abdomen** Mean C_t (± 95%CI)	**Thorax** Mean Ct (±95%CI)	**Head** Mean Ct (±95%CI)
No detectable/trace RNA	29	No Ct	No Ct	No Ct
RNA abdomen only	6	30.3 – 35.5	No Ct	No Ct
RNA thorax/abdomen	6	26.9 – 29.9	31.6 – 36.8	No Ct
RNA head/thorax/abdomen	18	25.9 – 26.9	26.2 – 27.8	29.1 – 31.3

4.3.4 Viral detection in the whole body of orally infected C. sonorensis *and* C. nubeculosus

A total of approximately 600 adult female *Culicoides* (300 *C. sonorensis* and 300 *C. nubeculosus*) orally infected with BTV-1 GIB2007/01 KC_3 (C_t: 16.74 Titre: 7.5 $log_{10}TCID_{50}$/ml) were processed. After the initial blood meal (Day 0 p.i.) the C_t value in orally infected insects ranged between 30.69 to 34.09. From days 7 to 11, 19 individuals (15.2%) of the *C. sonorensis* tested contained RNA loads that exceeded the lower 95% CI of the level previously found in the abdomen of membrane fed/infected and fully disseminated individuals (C_t value =28.95) (Figure 4.4). Infectious virus was isolated from all but one of these individuals. Three individuals (2.4%) produced C_t values that were lower than C_t 28.95 , but still contained more RNA than the upper 95% CI of the original blood meal from day0 p.i. individuals (C_t value 32.75), while 37 (29.6%) produced C_t values lower than C_t 32.75. Sixty six (52.8%) individuals tested negative for BTV RNA.

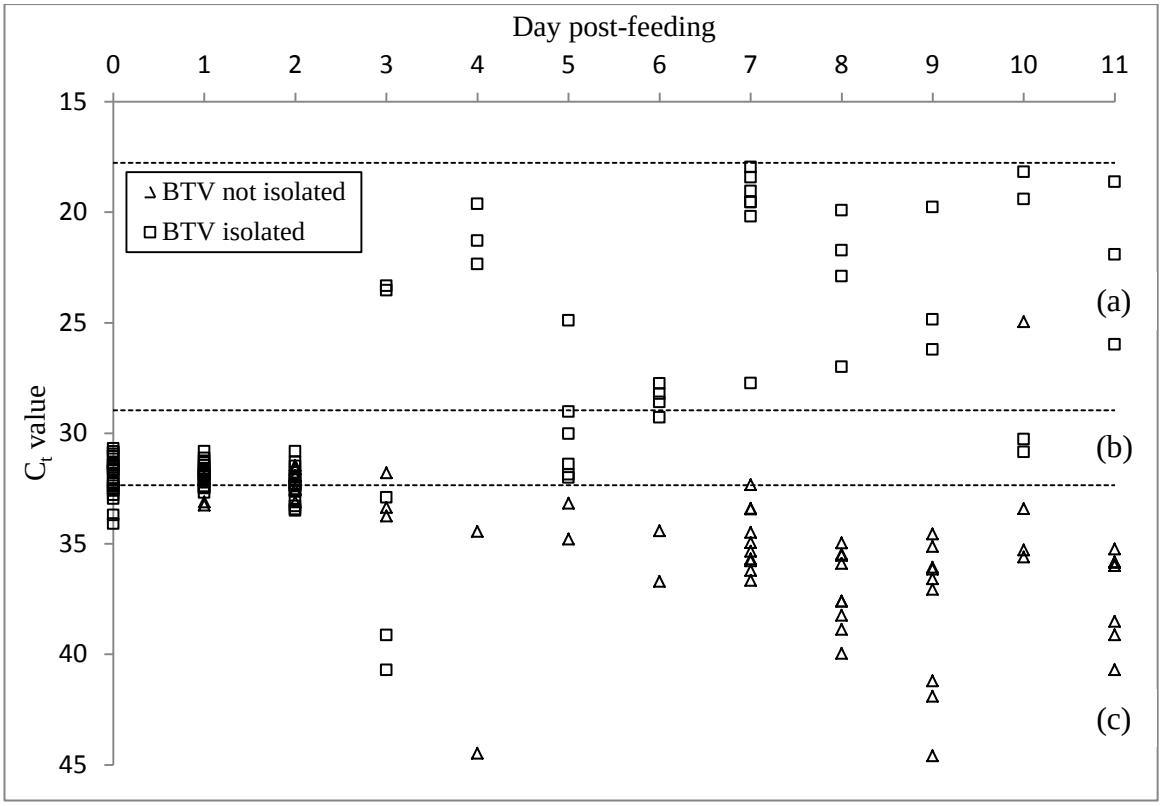

Figure 4.4: Detection of BTV RNA and infectious virus in orally infected *Culicoides sonorensis*

Following feeding on an infectious blood meal containing BTV-1 GIB2007/01 KC_3 (C_t: 16.74, titre: 7.5 $\log_{10}TCID_{50}$/ml) via a membrane based system. Adult *C. sonorensis* can be divided into those that are thought to contain (a) 'fully disseminated infections (17.77-28.95 C_t) (b) sub-transmissible infections (28.95-32.35) (C_t) and (c) sub-transmissibly infected or retained inactivated virus (32.35-45 C_t). Twenty five female were processed at each day interval from day 0 to day 11. The results here presented are inferred from their infectivity ($\log_{10}TCID_{50}$/ml) measured by virus titration on KC cells assay and viral RNA load (C_t) measured by rtRT-PCR.

Data from *C. nubeculosus* (Figure 4.5) show only one individual (0.8%) containing an RNA load that exceeded the lower 95% CI of quantities found in the abdomens of fully disseminated individuals (determined by injection). One other individual (0.8%) also produced a C_t value lower than this limit, but higher than the upper 95% CI of the original blood meal, and 9 (7.6%) produced C_t values lower than that of the original blood meal.

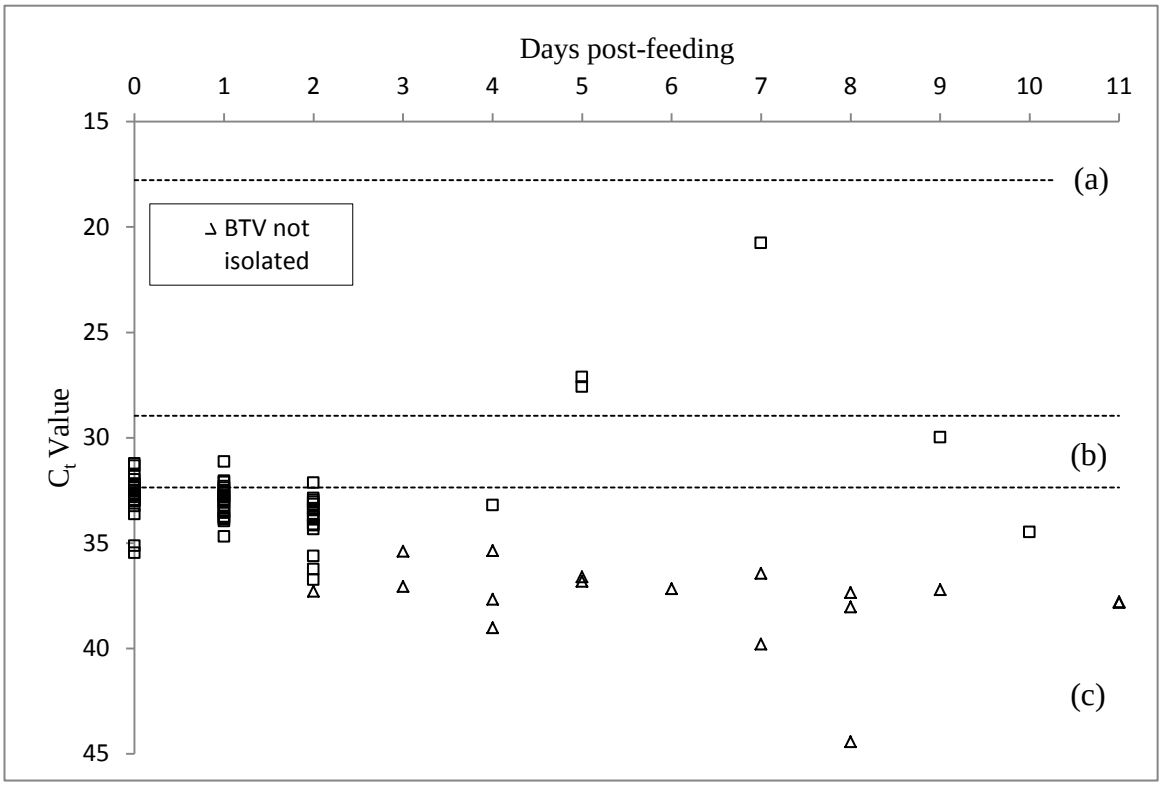

Figure 4.5: Detection of total BTV RNA and infectious virus in orally infected *C. nubeculosus*

Following feeding on an infectious blood meal containing BTV-1 GIB2007/01 KC_3 (C_t: 16.74, titre: 7.5 $log_{10}TCID_{50}$/ml) via a membrane based system, adult *C.nubeculosus* can be divided into those that are thought to contain (a) 'fully disseminated infections (17.77-28.95 C_t) (b) sub-transmissible infections and (28.95-32.35 C_t) (c) sub-transmissibly infected or retained inactivated virus (32.35-45 C_t). Twenty five female were processed at each day interval from day 0 to day 11. The results here presented are inferred from their infectivity ($log_{10}TCID_{50}$/ml) measured by virus titration on KC cells assay and and viral RNA load (C_t) measured by rtRT-PCR.

Table 4.6 shows the number of orally infected *C. sonorensis* female that were identified showing a virus titre ≥2.5 $log_{10}TCID_{50}$/ml on each day from day 0 to day 11 post infection. The highest and lowest virus titres in individual homogenates for each day interval is also given. The same table also shows the number of insects from the same experiment that had C_t value ≤30 and highest RNA / lowest C_t values recorded in individual insects at each day interval.

Table 4.6: Time course of *C. sonorensis* (oral) infection with BTV-1 GIB2007/01 KC_3.
Following feeding on an infectious blood meal containing BTV-1 GIB2007/01 KC_3 (C_t: 16.74, titre: 7.5 $log_{10}TCID_{50}$/ml) via a membrane based system, adult female *C. sonorensis* were incubated at 25°C for 11 days and analysed as a time series post-infection using rtRT-PCR and isolation on a KC cells.

d.p.i.	**Infectious BTV titre ($log_{10}TCID_{50}$/ml)**		**Viral RNA (Ct)**	
	C. sonorensis showing virus ≥2.5 $log_{10}TCID_{50}$/ml	Peak infectious BTV titre ($log_{10}TCID_{50}$/ml)	*C. sonorensis* with C_t values ≤28.95	Peak (lowest) C_t value/midge
0	25/25	>4.5	0/25	30.07
1	19/25	>4.5	0/25	30.82
2	7/25	>4.5	0/25	30.82
3	2/25	3.75	2/25	23.33
4	3/25	4.25	3/25	21.29
5	3/25	4.25	1/25	24.89
6	2/25	3.25	3/25	27.75
7	6/25	>4.5	7/25	17.96
8	4/25	>4.5	4/25	19.91
9	3/25	>4.5	3/25	19.77
10	2/25	4	3/25	18.18
11	3/25	4.25	3/25	18.62

In *C. sonorensis* the use of a C_t cut-off value of 28.95 (representing the upper 95% confidence limit for C_t value) for the abdomens of fully disseminated individuals, gave results that were consistent with the virus titration methods previously used, i.e. ≥2.5 $log_{10}TCID_{50}$/ml infectious virus (Table 4.6). In both techniques the peak of infectivity and virus RNA levels (lowest C_t values) were reached by day seven post-infection with similar predicted levels of competence.

4.3.5 Investigation of BTV dissemination in C. sonorensis *by confocal observation*

Preliminary confocal observation of *C. sonorensis* infected by intrathoracic inoculated with BTV-1 KC_2 (GIB 2007/01) confirmed the dissemination of BTV in the head, thorax and abdomen (Figure 4.6).

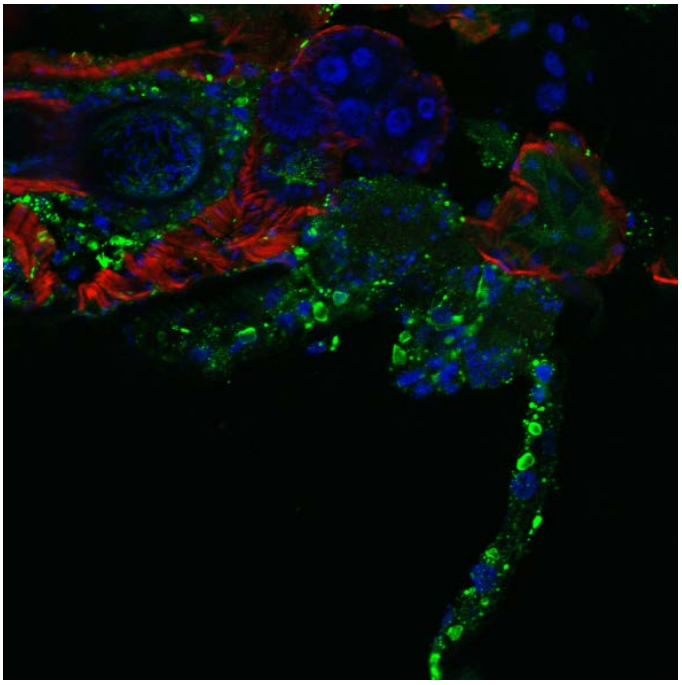

Figure 4.6: Confocal microscopy of *C. sonorensis* infected with BTV-1 GIB2007/01 KC_2. BTV-1 was detected in sections of *C. sonorensis* by staining for the non-structural protein NS2 (green), showing disseminated throughout the insect. BTV was present in the head (top right), thorax (middle top) legs (bottom right) and in the abdomen (top left). The cell nuclei are stained in blue (DAPI) and actin present in the muscles in red colour the (phalloidin labelled with fluorescent Alexa Fluor® 568).

4.4 Discussion

This chapter provides tools for assessing vector competence of three different species of *Culicoides* for BTV and forms a framework for future studies in this area. This is the first study to assess the potential for an rtRT-PCR assay to help incriminate *Culicoides* as BTV vectors and allows the interpretation of previous studies carried out before this validation. The implementation of this technique is important as rtRT-PCR now represents a 'front-line' tool to detect BTV that is used throughout reference

laboratories in Europe. It is more rapid and more readily reproducible and therefore far more likely to be used than the more traditional virus isolation methods. Two key criticisms of previous studies conducted using rtRT-PCR to implicate *Culicoides* as vectors of BTV have been an inability to differentiate live infectious virus from inactivated or low level 'sub-transmissible' infections from 'transmissible' ones (Carpenter *et al.*, 2008c; Carpenter *et al.*, 2009). These issues are addressed in this chapter, by both exploring and comparing results from both methodologies that can be used to assess dissemination and by comparing BTV isolation rates with C_t values. In addition, the KC cell line was assessed for its sensitivity for quantitatively defining the degree of BTV dissemination.

Initial dissections of IT inoculated *C. sonorensis* confirmed the lack of salivary gland barriers through the presence of infectious virus in the saliva of all individuals (Bowne & Jones, 1966; Fu *et al.*, 1999; Jennings & Mellor, 1987). In addition they demonstrated that BTV in the saliva of fully disseminated individuals could be detected using either the rtRT-PCR or isolation on KC cells, followed by AgELISA. The peak titre attained in body parts of these *C. sonorensis* represented the likely upper limit of replication within *Culicoides* for two reasons. Firstly, this species is the largest major vector of BTV worldwide, being almost double the size of *C. brevitarsis* (Wirth & Hubert, 1989) and 30% larger than *C. imicola* (Meiswinkel, 1989; Wittmann, 2000). Secondly, the inoculation method introducing a high concentration of BTV directly into the haemocoel is considered likely to result in greater overall replication that natural penetration of the gut-barriers to dissemination.

The subsequent use of membrane feeding provided a more realistic assessment of BTV replication and was applied to *C. imicola* as a model for smaller vector species. As expected, membrane-fed *C. sonorensis* and *C. imicola* possessing fully disseminated

infections contained less quantity of BTV RNA in all body parts than IT inoculated *C. sonorensis*. Interestingly, in the case of membrane-fed *C. sonorensis* a single individual was also found possessing low quantities of BTV RNA throughout the abdomen, thorax and head. This appears to indicate only partial replication, possibly associated with the haemocoel dissemination barrier previously described (Fu *et al.*, 1999). Time series infection studies with *C. sonorensis* and *C. nubeculosus* appear to show that these infections were not a result of a slower replication rate in some individuals as the proportion of fully disseminated individuals remained constant from day 7 to 11 post-infection.

In membrane fed individuals, the demonstration of infectious virus or viral RNA in the head, using either isolation of infectious BTV on KC cells, or rtRT-PCR respectively, shows a clear potential for the use of this technique to identify individual *Culicoides* that have a fully disseminated infection. Although saliva-based detection is generally considered to be the gold standard for demonstrating dissemination (Boorman, 1987; Bowne & Jones, 1966; Chandler *et al.*, 1985; Foster & Jones, 1973; Foster *et al.*, 1963; Luedke *et al.*, 1967), it gave less consistent results, and in membrane-fed individuals of *C. sonorensis* and *C. imicola,* appeared to be close to the limit of sensitivity of both the two assays used. When operating under 'glove-box conditions', the collection of saliva from adult *Culicoides* is a labour intensive and technically demanding and time-consuming process, and the method does not allow immediate confirmation that saliva collection has been successful. Although the presence of saliva globules during salivation induced by malathion solution was previously described (Fu, 1995), these were not observed during the current study.

While currently unsuitable for vector competence assessments, higher throughput saliva collection may be possible from *Culicoides* using the recently developed FTA®

card techniques which allow natural sugar feeding on a honey-coated surface (Hall-Mendelin *et al.*, 2010). To date, no studies have validated this method for use with *Culicoides*-borne pathogens, however, it is known that these insects will feed readily from sugar baits. A second key area of investigation is the relationship between the amount of BTV infectious particles present in the saliva of fully disseminated midges and its correlation with their infectivity for a susceptible host. This area is currently only poorly understood due to the difficulties of measuring total saliva transferred during the blood meal. However previous studies have indicated that even a single bite from an adult midge with a fully disseminated BTV infection will reliably transmit the virus (Foster *et al.*, 1968). This is suggesting that sufficient virus, in a 'suitably infectious form', must be present in even very small quantities of saliva from these fully infected individuals.

Considering the potential changes in the genetic make-up of the virus adapted to standard mammalian cells (Bando, 1975; Gard, 1987; Wechsler & Luedke, 1991; Wechsler & McHolland, 1988) comparing to field samples, the advantages of using an AgELISA to confirm virus replication in insect cells (KC cells) without pre-adaptation to mammalian cells (e.g. BHK-21) is likely to play an important role in understanding of vector competence. Its application for the detection of differences in infectivity levels achieved with two adapted BTV stains, in both mammalian and insect cell-based infectivity assays, is also demonstrated here. The results obtained from titration of a BTV strain that was adapted to KC cells (BTV-8 KC_5) shows low level of infection when directly titrated on BHK-21 cells (confirmed by CPE) but a much higher titre (more than 4 $\log_{10}$ $TCID_{50}$/ml) when tested using KC cells followed by the BTV specific AgELISA. Adaptation to growth in BHK-21 cells is likely to select viruses that can infect and be effectively released from BHK-21 cells (causing CPE), allowing them to infect other cells and grow to higher titres. Viruses which are adapted to BHK-21 cells may have

differences in their genome segments/proteins involved in cell release (e.g. Seg-5/NS1 and Seg-10/NS3/NS3a), that may affect their ability to infect, disseminate within and be transmitted by adult *Culicoides*.

The AgELISA, while being highly reliable and repeatable during assessment of cell culture titres in KC cells, was found to be limited in sensitivity at low titres of BTV in whole *Culicoides*, even following further passage on cells. This resulted in OD values very close to the lower threshold limit making interpretation of their positivity difficult. It is unclear what mechanism caused this problem. Practically, the impact of this observation was limited, as *Culicoides* containing fully disseminated and therefore higher levels of infections could still be clearly identified.

Analysis of 'RNA load' present over time within the whole body of membrane-fed *C. sonorensis* and *C. nubeculosus*, closely resembled published studies that utilized detection assays dependent upon live virus isolation (Foster & Jones, 1979; Fu *et al.*, 1999; Mullens *et al.*, 1995; Wittmann *et al.*, 2002). During an initial partial eclipse phase (where virus particles cannot be detected) (Foster & Jones, 1979) from 0 to 48h post infection, ingested BTV-1 RNA was cleared by the majority of individuals. Evidence of replication of BTV was inferred where the quantity of BTV-1 RNA in individuals, significantly exceeded that contained in the original blood meal, which was recorded at 72h incubation in *C. sonorensis* and 120h in *C. nubeculosus*, although lowest C_t values (peak viral RNA levels) were not reached in either species until 7 days post-feeding. The inferred range of competence for both *C. sonorensis* (ranging from 12-20% for days 7-11 post-infection) and *C. nubeculosus* (0-4%) agreed closely with previous studies of these colony strains, despite using (in the study described here) a KC passaged BTV strain and a detection system based on viral RNA rather than virus isolation (Jennings & Mellor, 1987; Mellor & Wittmann, 2002).

With regard to the assessment of field collected *Culicoides* for analysis of vector competence, the results described in this Chapter have wide implications. Taking the cohort of putatively infected *C. sonorensis* from days 7-11, some 70% of individuals that would have been reported as positive by conventional or unquantified rtRT-PCR did not possess a fully disseminated infection. It is unclear at present what proportion of these individuals possessed persistent sub-transmissible infections, or had retained inactivated BTV RNA following ingestion. It is clear, however, that the findings of previous studies using un-quantified rtRT-PCR or conventional RT-PCR-based methods to simply detect *Orbivirus* (or any other arbovirus) cannot be relied upon to demonstrate full vector competence in *Culicoides* species (Becker *et al.*, 2010; Dijkstra *et al.*, 2008; Mehlhorn *et al.*, 2007; Meiswinkel *et al.*, 2007; Sabio *et al.*, 2006). The detection and quantification of viral RNA will also vary both with the nature of the different rtRT-PCR assay being used (e.g. which genome segment it targets) and with the virus strain involved, and will therefore require standardization if reliable data comparison is to be achieved. Another important aspect that should be taken in consideration is the standardization of the current methods with smaller *Culicoides* species that can be consistently blood-fed. According to the results presented here, there is a difference in the level of BTV dissemination among *C. sonorensis* and *C. imicola,* which could indeed be explained by the smaller size of the latter, and hence fewer cells that may be infected. This may be particularly important as the number of cells involved could increase according to difference in mass; roughly the 'cube' (power of three) of the difference in size (length).

Finally, a preliminary study was conducted to examine the localisation of BTV non-structural viral protein (NS2) in the body of *C. sonorensis* intrathoracically inoculated with BTV-1 KC_2 (GIB 2007/01), and it confirmed the presence of NS2 by confocal imaging of immuno-labelled longitudinal sections of the insects. While

dissemination of BTV could be traced visibly in *C. sonorensis* examined, several difficulties were encountered in use of the technique. Firstly, despite embedding in O.C.T. compound the use of a cryostat technique to produce longitudinal sections was found to have some drawbacks including inconsistency in sectioning with damage to some areas of the specimen. The method, however, remained superior to previous studies where specimens risked being dislodged from the fixing media. Secondly background fluorescence was also documented during observation of samples and careful production of a negative control is important in future studies.

In conclusion, the data described in this Chapter have confirmed the validity of an AgELISA for the detection of virus amplification in an insect cells based assay. According to the specificity and sensitivity of the assay, a cut-off value ranged between 0.18-0.20 was here defined. The application of this assay was successfully demonstrated by detection of virus amplification in full disseminated *C. sonorensis*. The importance on validating this assay also allows confirmation of viral RNA amplification (by rtRT-PCR) in infected *Culicoides*. The data here presented are demonstrating for the first time, the potential for an rtRT-PCR to confirm full dissemination in orally infected *Culicoides*.

Despite only preliminary data, it has been here demonstrated the potential of confocal microscopy for the investigation of virus replication in infected *Culicoides* by detection of non-structural viral proteins (NS2) in cryosections of whole bodies.

Chapter 5: Estimating the thermal limit of replication and extrinsic incubation period (EIP) for BTV strains in adult *Culicoides* and KC cells

Virus replication of twelve different BTV strains were initially assessed by incubation at three different temperatures (12, 14 and 25ºC). Two of these strains that showed differences in thermal limits of BTV replication were then investigated in more detail by incubation at five different temperatures (8, 10, 12, 15, 20 and 25ºC). The extrinsic incubation period (EIP) of these strains was then also defined in orally infected *C. sonorensis* and *C. imicola* incubated at 15 and 25ºC. Replication on the KC C. sonorensis cell line was demonstrated to provide a rapid means of defining temperature-related differences in phenotype in BTV strains. In addition, the minimum threshold for replication in some BTV strains was shown to be between 10 and 12°C which was considerably lower than that detected in *Culicoides*. The implications of these results on the epidemiology of BTV are discussed.

5.1 Introduction

Arbovirus strains vary both in their ability to replicate at low temperatures and their speed of replication over a range of temperatures (Mellor, 2000; Mellor & Leake, 2000). This variation is a key parameter contributing to the wider epidemiology of the arboviruses, as the length of the extrinsic incubation period (EIP) in the vector is largely determined by environmental temperature (Hales *et al.*, 2002; Hardy, 1988; Reeves *et al.*, 1994; Turell *et al.*, 1985). While EIP is usually inversely related to ambient

temperature (Bates & Roca-Garcia, 1946; Kramer *et al.*, 1983; Mullens *et al.*, 1995; Wittmann, 2000), variation in enzyme-driven processes of the arbovirus itself and of the vector's metabolism, can lead to significant differences in cold and heat tolerance (Mertens *et al.*, 1987).

In regions possessing wide seasonal fluctuations in temperature, and in particular those with cold winters (e.g. Europe and North America), knowledge of the temperature dependence of the EIP can be used to model the probability of virus establishment and onward transmission following an incursion into a specific area and at a specific time of the year (Wilson & Mellor, 2008). These data can also be incorporated into more refined risk models to assist in disease outbreak management and planning e.g. to assess the probable timing of disease recrudescence in spring (Carpenter *et al.*, 2011; Gloster *et al.*, 2008; Gubbins *et al.*, 2008; Purse *et al.*, 2005; Wittmann & Baylis, 2000).

Systematic studies of the relationship between temperature and EIP have been carried out for several arbovirus-vector relationships. These include the replication of AHSV (Wellby *et al.*, 1996), EHDV and BTV in colony derived *C. sonorensis* (Mullens *et al.*, 2004; Mullens *et al.*, 1995; Paweska *et al.*, 2002; Wittmann *et al.*, 2002). Transmission of several mosquito transmitted diseases in relation to temperature has also been intensively investigated, including Western equine encephalitis virus and St Louis encephalitis virus in *Ae. tritaeniorhynchus* (Hardy *et al.*, 1990; Reisen *et al.*, 1993), Rift valley fever virus in *Culex pipiens* (Turell, 1993), dengue haemorhagic fever virus (Lambrechts *et al.*, 2011; Watts *et al.*, 1987) and Okelbo virus (Lundstrom *et al.*, 1990; Turell & Lundstrom, 1990) in *Aedes albopictus* and West Nile virus in *Culex pipiens* (Dohm *et al.*, 2002; Kilpatrick *et al.*, 2008; Reisen *et al.*, 2006; Richards *et al.*, 2007).

The methodologies used in studies of the EIP vary significantly, but usually involve the infection of either vector-associated cell lines or large numbers of colony-reared model vectors. The infected cells or vectors are then incubated at constant temperatures and tested for arbovirus content at pre-defined time intervals post-inoculation or blood-feeding. These studies are usually time consuming and labour intensive, particularly where the model vector competence for the arbovirus being studied is low. An important example is provided by previous studies of the replication of two BTV strains in *C. sonorensis* (Wittmann *et al.*, 2002). Only ten individuals of *C. sonorensis* were processed at each time period and at each temperature following feeding, but a competence rate of approximately 10% for both the BTV strains led to results that were difficult to interpret.

In addition to the logistical difficulties in processing large numbers of individual arthropods, there are also several other methodological limitations in previous studies of the EIP of different BTV strains. Firstly, most of the readily available BTV strains had been isolated and passaged repeatedly in mammalian cell cultures (Fu *et al.*, 1999; Gard, 1987; Jennings & Mellor, 1987; Mellor & Boorman, 1980) or in embryonated hens eggs (Jones & Foster, 1971). This methodology supported the use of straightforward detection techniques following processing of *Culicoides* (such as observation of CPE in infected BHK cells) which is a significant advantage when processing a large number of individuals. However, this approach has been criticized, as the temperature requirements and growth characteristics of highly passaged BTV strains may be different from those of field strains (Mullens *et al.*, 2004). Secondly, carrying out EIP determination with colony *C. sonorensis* that are adapted to their maintenance temperature of 27°C, imposes difficulties in examining low temperatures (≤15°C), as increasing mortality rates and longer EIP's make direct determination of lower

threshold replication temperatures of BTV strains unachievable. Thirdly, at higher temperatures where replication is rapid (25-30°C) and the whole dissemination cycle could take as little as few days, it becomes increasingly problematic to separate excretion of BTV in the original blood meal from newly replicating virus in *C. sonorensis*. This prevents straightforward estimations of BTV replication rate to full dissemination levels, at higher temperatures.

One of the primary aims of the current study was to establish the lower thermal limits for replication and transmission by *Culicoides* vectors, for selected BTV strains. This would allow re-evaluation of the paradigm used during the 2007 BTV UK outbreak, that replication would not take place at temperatures lower than 12°C (Mullens *et al.*, 1995; Paweska *et al.*, 2002; Wittmann & Baylis, 2000). To accomplish this it was necessary to establish rapid, sensitive, reproducible and convenient methodologies for detecting and quantifying virus replication at different temperatures.

Previous studies have established that cell lines derived from *C. sonorensis* embryos (CuVa 1-8) developed highest titres of virus when infected with BTV-11 (passage 13 times in BHK-21 cells) at 25 and 32°C (with lower titres developed at 20 and 37°C) (Wechsler *et al.*, 1989) . Additionally, low passage strains of BTV induced CPE when infected with BTV, but this was not affected by temperature (See also Chapter 6). Differences have been reported in the CPE induced by BTV in Bovine Kidney cells (MDBK) at different temperatures (Hallum *et al.*, 1984). It was observed that cells became persistently infected when incubated at 37°C, but were lysed after the cells were shifted to 30°C. The highest BTV titres recorded by Weschler et al. (Wechsler *et al.*, 1989) were also very close to the optimum temperature recorded for BTV polymerase activity by Verwoerd and Huismans (Verwoerd *et al.*, 1972) . So far, no information is available concerning any differences in the optimal temperature of the isolated

polymerase of different BTV isolates, or their optimal temperature for replication in infected cells or tissues.

These considerations make KC cells a candidate for studies of intracellular BTV replication, over a wide temperature range, to help determine both the thermal limits and assess the effects of temperature on virus transmission. Following the initial development of these techniques, using KC cells and related assay systems (Chapter 4), it was necessary to compare them against more traditional methods, using feeding of blood/BTV mixtures to laboratory reared *C. sonorensis*. In this way it was possible to evaluate and validate the replication of BTV in these cells, as a model for the behaviour of the pathogen in vector insects.

5.2 Materials and Methods

5.2.1 The replication of 12 selected BTV strains in KC cells

The potential for replication of BTV strains, at a series of temperatures, in KC cells was assessed for twelve strains of BTV (Table 5.1). Full taxonomic details of each BTV strain are available at http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm and Chapter 2.1, Table 2.1. The initial titre of each BTV reference strain was determined by serial dilution (from 10^{-1} to 10^{-7}) on KC cells (Chapter 2, Section 2.4.2). After 7 days incubation, 50µl of supernatant from each well of the infected plate was tested for confirmation of virus replication, by Ag-ELISA (Chapter 4, Section 4.2.1). According to the results obtained, each BTV strain was then diluted in Schneider's media, to a final virus titre of 5.0 $Log_{10}TCID_{50}$/ml. Where strains had a virus titre lower than 5.0 $Log_{10}TCID_{50}$/ml, a further passage in KC cells was used to amplify the virus, which was then titrated and diluted to the final concentration (Table 5.1).

Replication of each BTV strain was assessed at 12, 15 and 25°C. Two hundred microliters of each BTV strain were inoculated into 25 cm^2 flasks containing 10 ml of growth media and re-suspended KC cells (Chapter 2, Section 2.1.2). Two duplicate flasks were produced for each strain and temperature. Hence, 48 flasks were incubated at 12 or 15°C for 4 weeks, while 24 flasks were incubated at 25°C for 3 weeks only, due to deterioration of the cell layer if kept longer. Following this incubation period, supernatant from each flask was tested for virus replication by titration on the KC cells (Chapter 2, Section 2.4.2) followed by AgELISA (Chapter 4, Section 4.2.1). Samples were also tested directly using rtRT-PCR to determine the quantity of BTV RNA generated, to confirm the results and accuracy of the Ag-ELISA technique (Chapter 2, Section 2.5.1, 2.5.2).

Table 5.1 Bluetongue virus strains tested for replication at different temperatures in the KC *C. sonorensis* cell line.

The geographic origin of each strain (topotype) is abbreviated as "W" for western and "E" for eastern. The number of each passage is indicated with a subscript number after each cell line.

* Titre of the original virus isolate from the reference collection. **Virus titre of the sample after amplification in KC cells for 7 days at 25°C.

Strain description	Topotype	Passage history	Virus titre* ($Log_{10}TCID_{50}$/ml)	Passage history	Virus titre**($Log_{10}TCID_{50}$/ml)
BTV-9 LIB2008/01	W	KC_1	2.5	KC_2	5
BTV-9 BOS2002/02	E	E_1BHK_3	5.25	$E_1BHK_3KC_1$	5.5
BTV-4 SPA2004/02	W	E_1BHK_1	4.25	$E_1BHK_1KC_1$	6
BTV-2 SAD2002/02	W	E_1BHK_2	5	$E_1BHK_3KC_1$	6.25
BTV-16 ISR2009/01	E	KC_2	6.5	KC_3	6.75
BTV-16 SAD2004/02	E	BHK_2	5.5	BHK_2KC_1	6.5
BTV-1 GRE2001/01	E	BHK_3	4	BHK_3 KC_1	7
BTV-1 GIB2007/01	W	KC_1	2.5	KC_2	6.5
BTV-8 NET2008/03	W	$E_1BHK_2KC_1$	6.25	$E_1BHK_2KC_1$	6.25
BTV-1 FRA2007/18	W	BHK_1	6.0	BHK_1KC_1	6.0
BTV-8 DEN2008/01	W	KC_1	5.5	KC_3	7.75
BTV-8 UKG2007/06	W	KC_5	8	KC_5	8

5.2.2 Replication of two selected BTV strains in KC cells

Following the screening of 12 BTV strains for replication characteristics, two BTV strains were selected and compared in a more detailed time-series analysis. A total of ten 75cm^2 flasks containing resuspended KC cells were inoculated with 200 µl of the two BTV strains (as for 5.2.1). The infected flasks were incubated at 5 different temperatures: 8, 10, 12, 15 and 25 °C. One ml of supernatant was collected from each flask at daily intervals from day 0 to day 7 post infection (p.i.) then every second from day 7 to day 30 p.i. and stored in a 1.5ml tube at +4ºC until further tests. Infection of KC cells, measurement of virus replication and viral RNA quantification were carried out as described in Section 5.2.1.

Further statistical analysis of strain replication was conducted by Dr Simon Gubbins of the Mathematical Biology group at Pirbright (IAH). The viral replication data (either $\log_{10}$ $TCID_{50}$ or C_t values) were modelled using logistic growth curves with a common temperature-dependent replication rate. Parameters in the model were: initial virus level; maximum titre ($\log_{10}$ $TCID_{50}$) or minimum C_t value; replication rate; and threshold temperature for replication. The differences between strains was incorporated by assuming a hierarchical structure in the parameters, such that the parameters for each strain are drawn from higher-order distributions.

5.2.3 Replication of two selected BTV strains in C. sonorensis

The two BTV strains characterized in Section 5.2.2 were assessed for replication characteristics in adult *C. sonorensis*. Two to three day old laboratory reared *C. sonorensis* were orally infected with a mixture containing 2 ml of defibrinated horse blood and 2 ml of tissue culture supernatant from KC cells infected with one of the

BTV strains. The final concentration of the virus in the blood mixture for both strains was the same as used for *in vitro* infection in KC cells: 5.0 $\log_{10}$TCID$_{50}$/ml (5.2.1).

Approximately 2,200 adult female *C. sonorensis* were fed with blood / virus mixtures, containing either BTV-1w (GIB2007/01) or BTV-4w (SPA2004/02). Fully engorged insects were then selected under light CO_2 anaesthesia then incubated at 15 or 25ºC with 85-90% relative humidity for 30 or 8 days respectively. A total of 400 insects were processed for each virus strain at each temperature (as pools of 25 insects / day). Twenty five individuals of each species infected with each strain were collected at daily intervals, from day 0 p.i. to day 8 p.i. for insects incubated at 25ºC and 85-90% relative humidity, or every 5 days until day 30 p.i. for specimens incubated at 15ºC (the limit of survival). For each time point, the 25 insects were pooled in a 1.5 ml eppendorf tube, then homogenized (Chapter 2, Section 2.3 and Chapter 3). Virus replication was then assessed by titration of each homogenate on KC cells (Chapter 2, Section 2.4.2) followed by AgELISA (Chapter 4, Section 4.2.1). Viral RNA from each homogenate was also extracted and processed for BTV RNA quantification by rtRT-PCR (Chapter 2, Section 2.5.1, 2.5.2).

5.2.4 Infection and dissemination rates for pooled C. imicola *orally infected with BTV at 15 and 25ºC*

A total of 14,800 adult female wild caught *C. imicola* from South Africa, were orally infected with either BTV-1w (GIB2007/01) or BTV-4w (SPA 2004/02) (Chapter 2, Section 2.2.2). Engorged insects were collected into unwaxed paper cup (50 female/cup) and incubated at 15 and 25ºC with 85-90% relative humidity. Pools of 25 insects were processed at daily intervals (for insects incubated at 25ºC), or every 5 day interval (for insects incubated at 15°C) until days 8 or 30 p.i. respectively. A total of 325 adult insects were processed for each virus strain, for quantification of viral RNA

by rt-RT-PCR (See Chapter 2, Section 2.5.1, 2.5.2). Despite the use of antibiotics and fungizone at twice the standard concentration (Chapter 2, Section 2.1) contamination with fungus and bacteria, was a significant issue and precluded the isolation of infectious BTV.

Further investigation of viral RNA (C_t) was carried out with adult female *C. imicola* orally infected with BTV-1w and BTV-4w (using identical methods as described in Section 5.2.3), but processed individually (25 insects/day). After oral infection with either BTV-1w (GIB2007/01) or BTV-4w (SPA2004/02), fully engorged adult female *C. imicola* were incubated at 25ºC and 85-90% relative humidity for up to 8 days. Each day, a total of 25 insects were individually homogenized and titrated on KC cells ($log_{10}TCID_{50}$/ml) and viral RNA concentrations assessed by rtRT-PCR (C_t) as described above for the pooled insects.

5.3 Results

5.3.1 Replication of 12 BTV strains in KC C. sonorensis cells

Of the 12 BTV strains examined, three showed evidence of replication at 12°C. BTV-2 from Sardinia (SAD2002/02), BTV-1 from Gibraltar (GIB2007/01) and BTV-9 from Bosnia (BOS2002/02) developed mean virus titres of 6.13, 6.63 and 6.00 $log_{10}TCID_{50}$/ml respectively, with starting titre of 4.0 $log_{10}TCID_{50}$/ml. These results were confirmed by rt-RTPCR, which generated mean C_t values of 20.48, 18.12 and 20.61 respectively (Table 5.2 and Figure 5.1; 5.2). Among these strains, BTV-1 from Gibraltar, was the most epidemiologically the most interesting one as moved further north in Europe while the other two, were never found in northern altitudes. Other BTV strains tested showed no significant activity at 12°C

including BTV-8 strains originating from the UK, Holland and Denmark. At 15°C and 25°C all BTV strains tested demonstrated at least some degree of replication both in terms of the virus-titre detected and RNA production. Most of the BTV strains showed similar replication patterns at 25ºC, with virus titres between 6.0 and 6.63 $log_{10}TCID_{50}$/ml; apart from BTV-2 SAD2002/02 ($E_1BHK_3KC_1$) which generated the lowest virus titre, of 5.75 $log_{10}TCID_{50}$/ml and BTV-8 UKG2007/06 (KC_2) with a virus titre of 8.13 $log_{10}TCID_{50}$/ml (Table 5.2 and Figure 5.1, 5.2).

Table 5.2: Virus titre and RNA quantity (C_t) in the supernatant of BTV infected KC *C. sonorensis* cells at 12, 15 and 25°C.

BTV Strain*	12°C (day 28 post-infection)						15°C (day 28 post-infection)						25°C (day 21 post-infection)					
	Virus titre ($log_{10}TCID_{50}$/ml)			rtRT-PCR (Ct)			Virus titre ($log_{10}TCID_{50}$/ml)			rtRT-PCR (Ct)			Virus titre ($log_{10}TCID_{50}$/ml)			rtRT-PCR (Ct)		
	1st	2nd	Mean	1st	2nd	Mean	1st	2nd	Mean	1st	2nd	Mean	1st	2nd	Mean	1st	2nd	Mean
BTV-9 KC2 LIB2008/01	5.00	5.25	**5.13**	36.76	33.04	**34.90**	5.75	6.00	**5.88**	32.80	33.52	**33.16**	6.25	5.75	**6.00**	32.07	29.52	**30.80**
BTV-9 BOS2002/02	6.50	5.50	**6.00**	20.84	20.38	**20.61**	6.50	6.25	**6.38**	18.99	18.14	**18.57**	6.25	6.50	**6.38**	17.94	17.48	**17.71**
BTV-4 SPA2004/02	3.50	4.50	**4.00**	26.99	23.64	**25.32**	6.75	7.00	**6.88**	19.43	17.85	**18.64**	6.25	6.50	**6.38**	17.62	18.60	**18.11**
BTV-2 SAD2002/02	5.25	7.00	**6.13**	21.57	19.39	**20.48**	7.25	6.75	**7.00**	17.05	15.02	**16.04**	5.50	6.00	**5.75**	16.55	14.42	**15.49**
BTV-16 ISR2009/01	3.25	4.25	**3.75**	28.09	26.12	**27.11**	7.25	7.75	**7.50**	18.01	18.86	**18.44**	5.75	7.00	**6.38**	17.30	17.17	**17.24**
BTV-16 SAD2004/02	5.00	6.25	**5.63**	24.29	20.83	**22.56**	7.25	7.75	**7.50**	16.57	18.31	**17.44**	5.75	6.25	**6.00**	18.40	18.80	**18.60**
BTV-1 GRE 2001/01	6.25	5.50	**5.88**	27.58	23.33	**25.46**	7.00	7.25	**7.13**	20.90	20.23	**20.57**	6.00	6.00	**6.00**	22.21	21.81	**22.01**
BTV-1 GIB2007/01	6.25	7.00	**6.63**	18.55	17.69	**18.12**	7.25	7.25	**7.25**	16.32	17.15	**16.74**	6.75	6.50	**6.63**	16.66	17.38	**17.02**
BTV-8 NET2008/03	4.00	4.75	**4.38**	25.01	22.89	**23.95**	5.50	7.25	**6.38**	18.84	18.10	**18.47**	5.75	6.25	**6.00**	16.03	14.74	**15.39**
BTV-1 FRA2007/18	4.50	4.75	**4.63**	22.59	22.60	**22.60**	6.25	6.25	**6.25**	17.16	17.12	**17.14**	6.00	6.25	**6.13**	16.05	15.95	**16.00**
BTV-8 DK2008/01	4.25	4.00	**4.13**	20.71	20.82	**20.77**	5.50	5.75	**5.63**	18.95	18.94	**18.95**	6.50	6.50	**6.50**	13.42	14.14	**13.78**
BTV-8 UK2007/06	4.25	4.25	**4.13**	22.90	26.00	**24.45**	6.25	6.50	**6.37**	15.41	15.36	**15.39**	8.00	8.25	**8.13**	14.44	14.72	**14.58**

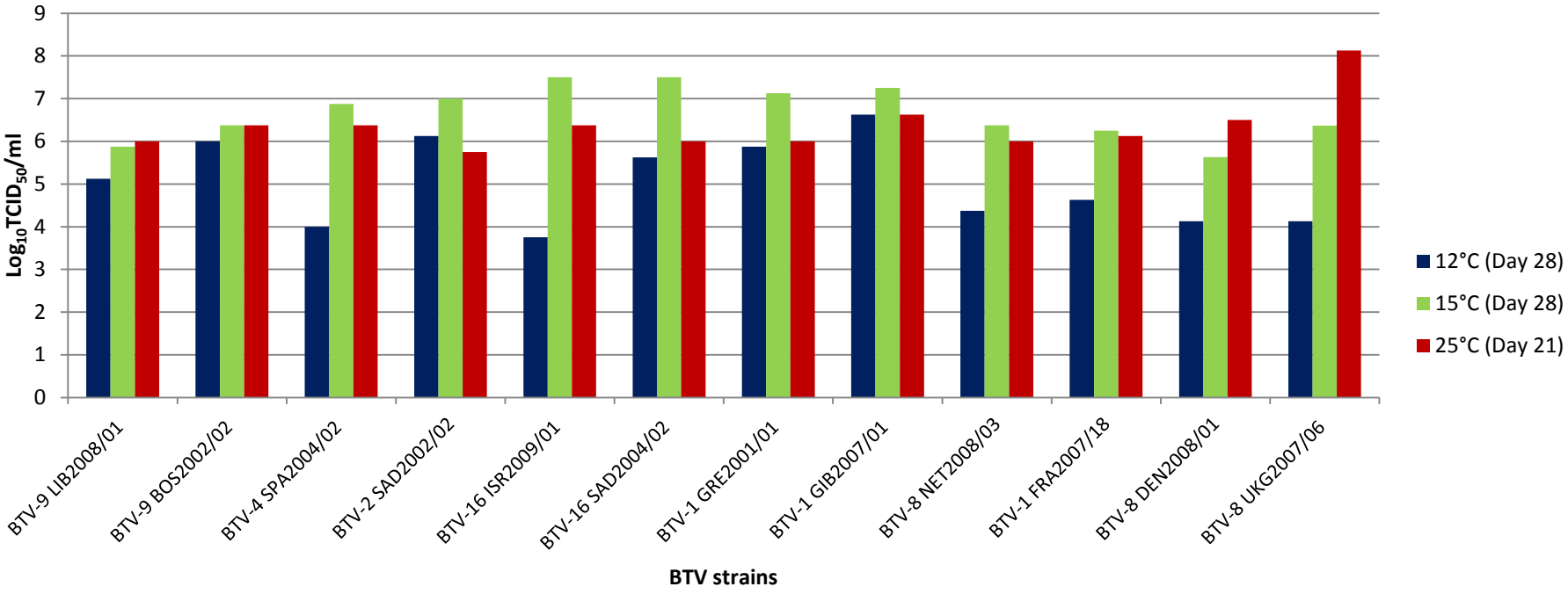

Figure 5.1: Virus replication ($log_{10}TCID_{50}$/ml) of BTV strains in KC-cell cultures following incubation at three temperatures (12, 15 and 25ºC). Supernatant frrm each incubated sample was tested as a ten-fold serial dilution on KC cells assay and virus titre confirmed by AgELISA.

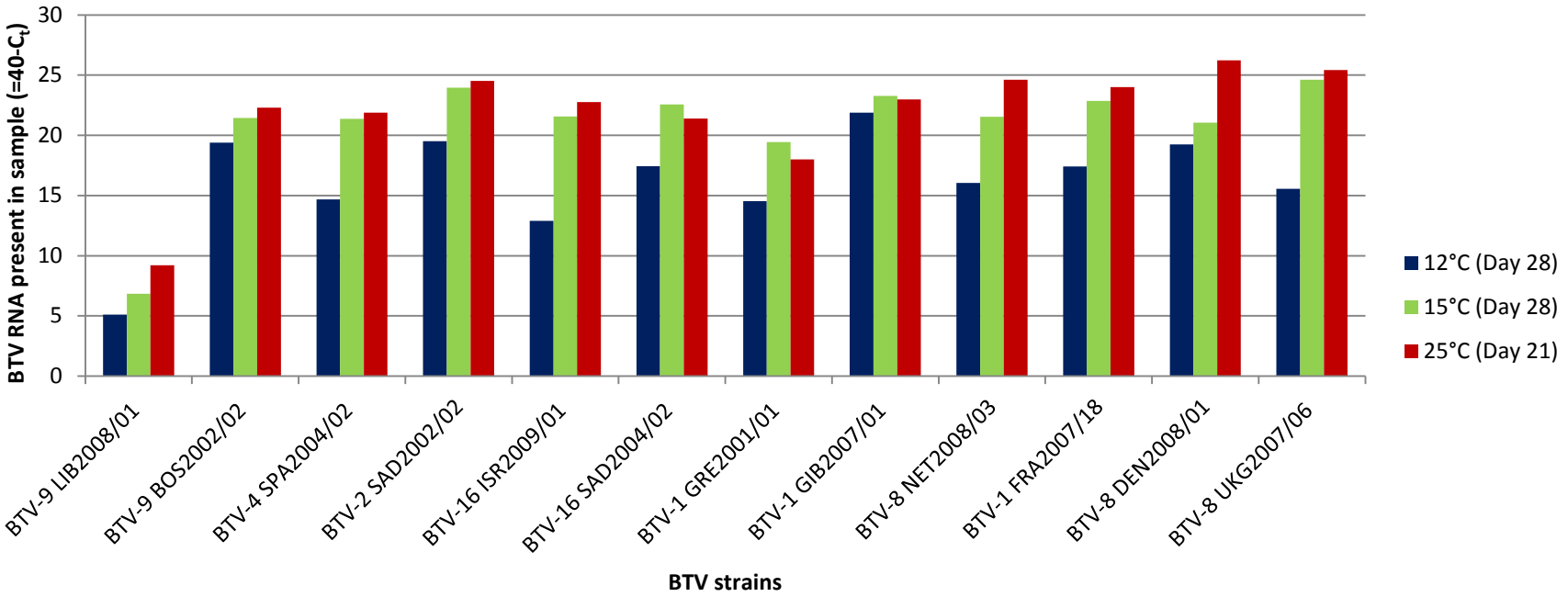

Figure 5.2: Viral RNA (C_t) of BTV strains in KC-cell cultures following incubation at three temperatures (12, 15 and 25ºC).
RNA was extracted from each supernatant and tested by rtRT-PCR. The C_t value recorded at the end of each PCR cycles (40) were subtracted from $40C_t$; the values here shown are therefore the final results of the subtraction and indicative of viral RNA increase.

5.3.2 The replication of two selected BTV strains in KC cells

Based on Section 5.3.1, BTV-1 GIB2007/01 (KC_2) and BTV-4 SPA2004/02 ($E_1BHK_1KC_1$) were selected as representative strains that either replicated, or did not replicate at 12°C (Table 5.2; Figs 5.1 and 5.2). Virus replication and BTV RNA production for the BTV-1 strain was confirmed at 12°C (Figure 5.3), although no evidence was detected for replication at 10 or 8°C. At day 0, infectious virus titres for duplicate flasks of BTV-1 were 1.75 and 2.5 $\log_{10}TCID_{50}$/ml, with C_t values of 28.71 and 30.93 respectively. The first major detectable increase in virus titre for BTV-1 incubated at 12°C was at day 11 p.i. (3.0-3.5 $\log_{10}$ $TCID_{50}$/ml), continuing to amplify to 4.5 $\log_{10}$ $TCID_{50}$/ml by Day 25 p.i. These results were mirrored by C_t values, although the increase in BTV RNA was not as evident as in the infectivity of the virus. At 15°C (Figure 5.4), infectious BTV again increased substantially at day 11 (to 4.5 $\log_{10}$ $TCID_{50}$/ml), although an increase in BTV RNA production was not evident until day 15 pi. Infectious virus production reached a plateau by day 21 p.i., of 7.25 $\log_{10}$ $TCID_{50}$/ml. At 25°C (Figure 5.4). There was broad correlation between peaks in infectious BTV and RNA production and infectious virus production which peaked and reached a plateau simultaneously at day 5 pi.

In contrast and confirming the results of 5.3.1, there was little indication of an increase in virus titre for BTV-4 SPA2004/02 at 12°C (Figure 5.5), although a slight decrease in C_t value indicated that some RNA synthesis, possibly due to slower replication, was also occurring at this temperature. Differences in virus amplification between the two BTV strains were also evident at warmer temperatures. At 15°C (Figure 5.6) BTV-4 replicated more slowly, increasing from day 11 (3.0/2.75 $\log_{10}$ $TCID_{50}$/ml) until the end of the experiment (at 6.5/5.5 $\log_{10}$ $TCID_{50}$/ml) and did not reach an obvious

plateau. The two strains were comparable in replication rate at 25°C (Figure 5.6) reaching a plateau (6.5 $\log_{10}$ $TCID_{50}$/ml) after 4 days p.i.

Analyses of the viral RNA load in supernatants from KC cells infected with BTV-1 and BTV-4 then incubated at the five different temperatures (Table 5.3 and Figure 5.3-5.6), showed no increase in viral RNA at 8 and 10°C, compared to the initial values measured at day 0 p.i.. The BTV-4 infected cells incubated at 12°C showed a slight increase of about 2-3 C_t value by day 23 to 25 p.i. (22.95/21.8 and 21.51/22.1 respectively). Supernatant from cells infected with BTV-1 also showed a clear increase in viral RNA at 12°C (Figure 5.3), starting at day 18 p.i. (27.85 / 27.11 C_t) reaching a maximum at day 30 p.i. (C_t values of 22.89/23.54).

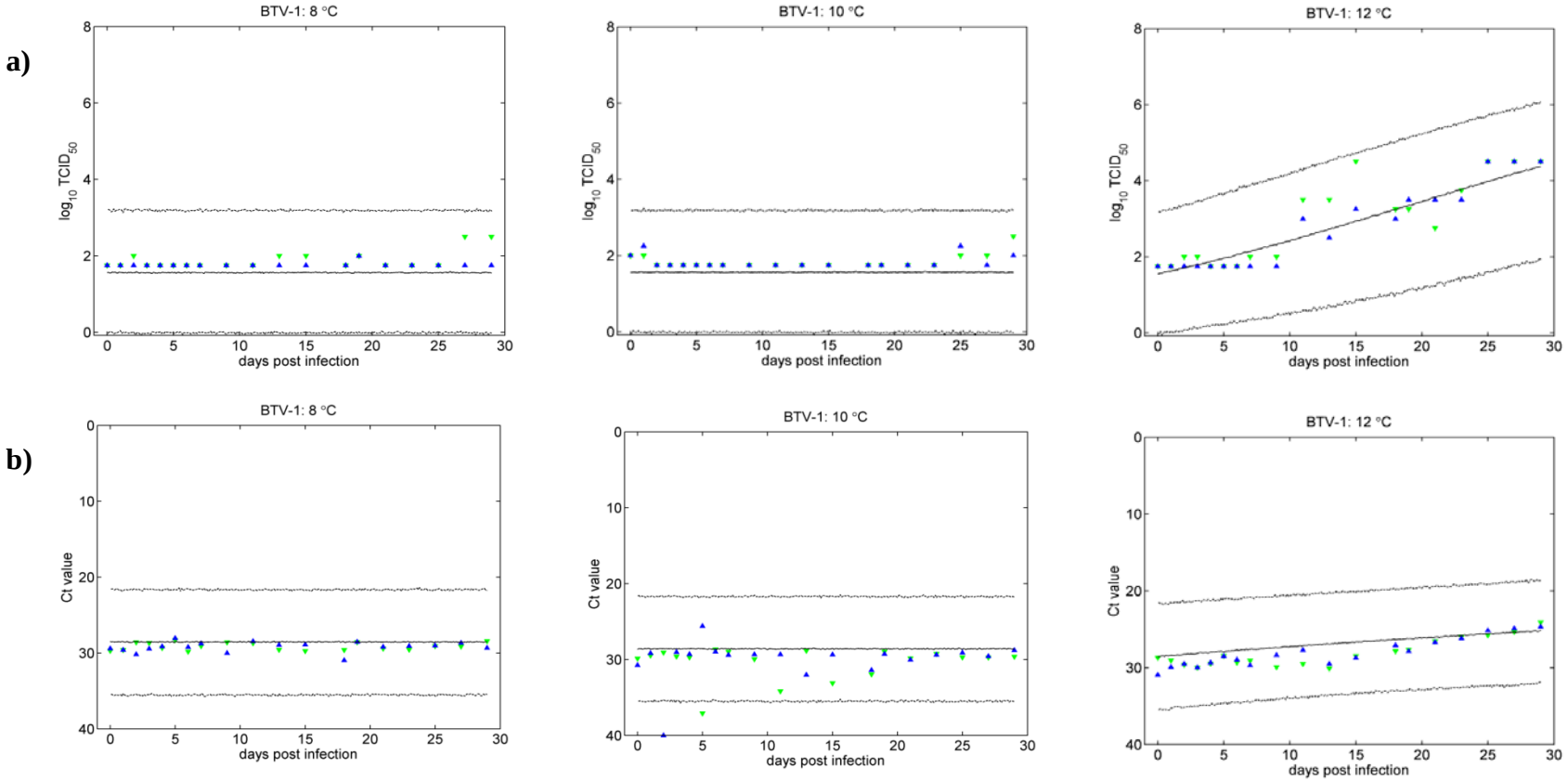

Figure 5.3: Observed and predicted changes in virus titres ($log_{10}TCID_{50}$/ml) (a) and viral RNA (C_t) (b) over time for BTV-1 GIB 2007/1 when incubated in KC cells at 8, 10 and 12ºC. Each figure shows the observed titre and C_t values for two flasks (green and blue triangles) and the posterior median (solid line) and 95% credible interval (dashed lines) for the predicted titre (Section a upper graphs) and C_t (Section b lower graphs).

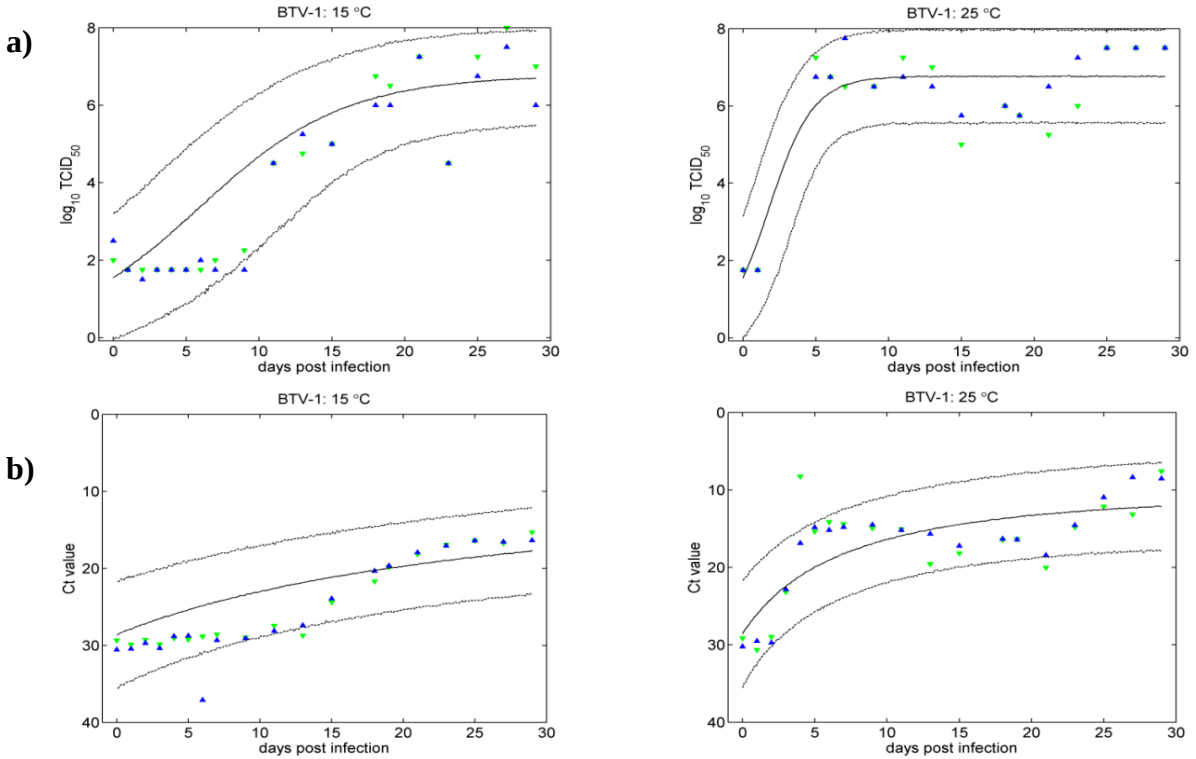

Figure 5.4: Observed and predicted changes in virus titres ($log_{10}TCID_{50}$/ml) (a) and viral RNA (C_t) (b) over time for BTV-1 GIB 2007/1 when incubated in KC cells at 15 and 25ºC. Each figure shows the observed titre and C_t values for two flasks (green and blue triangles) and the posterior median (solid line) and 95% credible interval (dashed lines) for the predicted titre (Section a upper graphs) and C_t (Section b lower graphs).

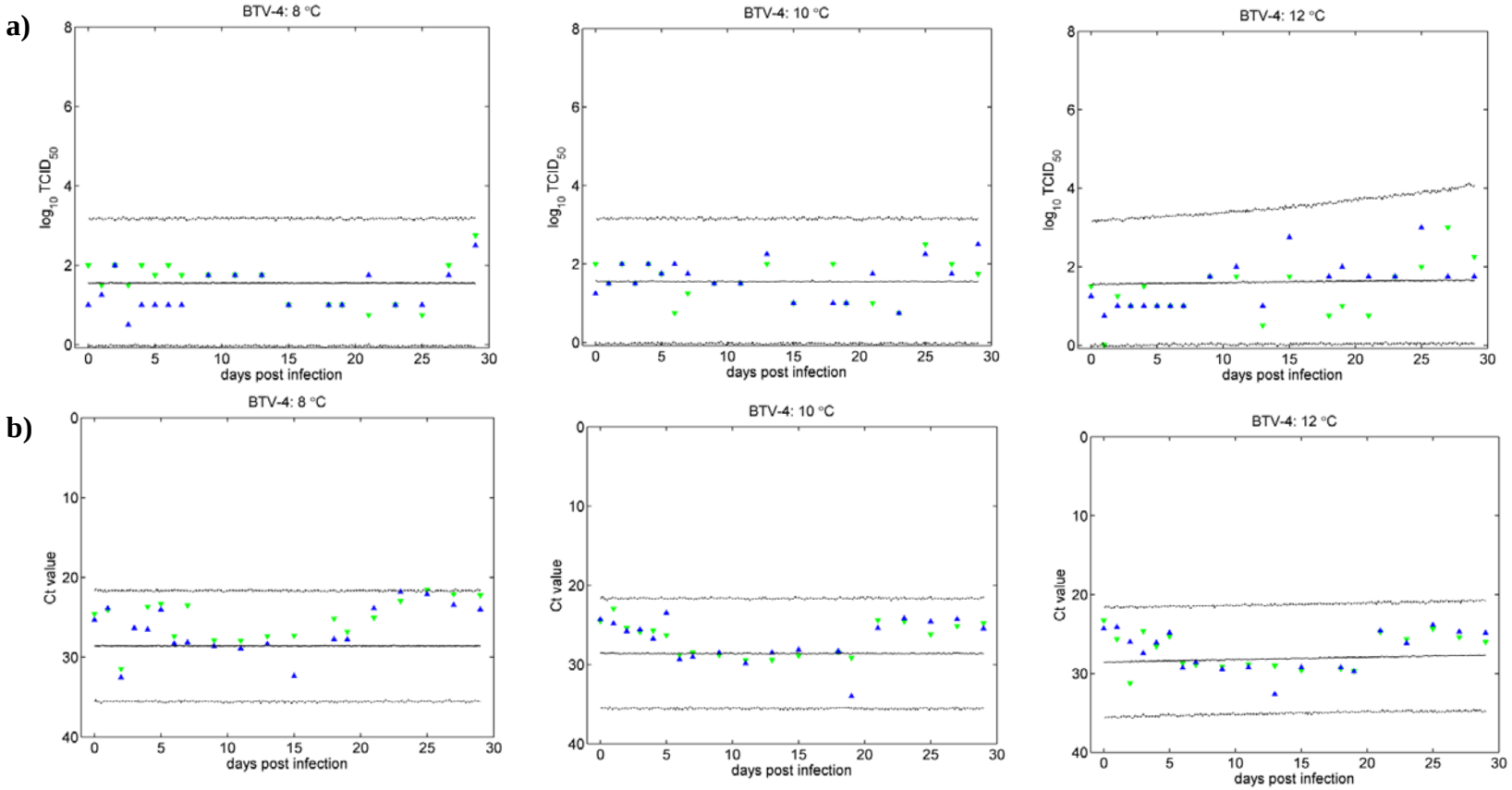

Figure 5.5: Observed and predicted changes in virus titres ($log_{10}TCID_{50}$/ml) (a) and viral RNA (C_t) (b) over time for BTV-4 SPA 2004/02 when incubated in KC cells at 8, 10 and 12ºC. Each figure shows the observed titre and C_t values for two flasks (green and blue triangles) and the posterior median (solid line) and 95% credible interval (dashed lines) for the predicted titre (Section a upper graphs) and C_t (Section b lower graphs).

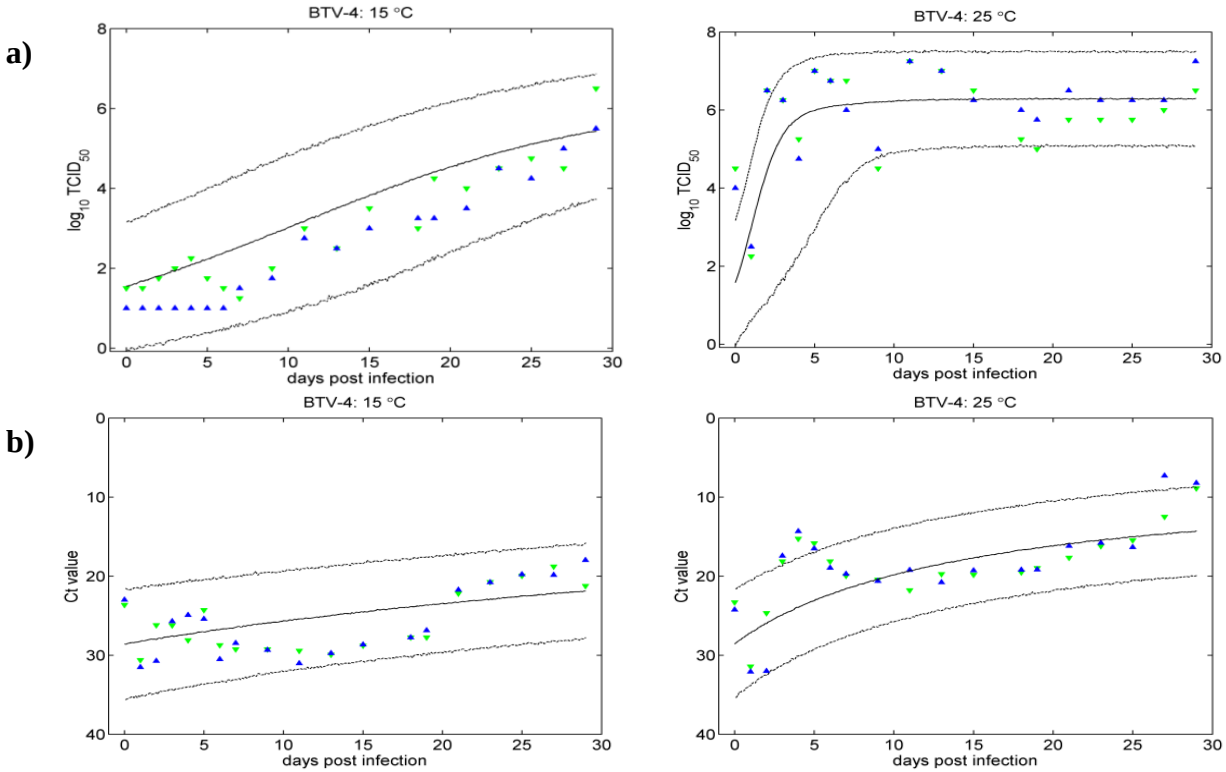

Figure 5.6: Observed and predicted changes in virus titres ($log_{10}TCID_{50}$/ml) (a) and viral RNA (C_t) (b) over time for BTV-4 SPA 2004/02 when incubated in KC cells at 15 and 25ºC. Each figure shows the observed titre and C_t values for two flasks (green and blue triangles) and the posterior median (solid line) and 95% credible interval (dashed lines) for the predicted titre (Section a upper graphs) and Ct (Section b lower graphs).

Analysis of infectivity ($log_{10}TCID_{50}$/ml) variations among the two strains (BTV-1 GIB2007/01 and BTV-4 SPA2004/02) were analysed by using the mathematical model explained in Section 5.2.2. (Appendix 7). The results shown in Table 5.3 and Figure 5.7 suggest that there may be differences between the strains in both threshold temperature for replication (BTV-1<BTV-4) and the virus replication rate above this threshold (BTV-1>BTV-4). However, there is considerable uncertainty in the estimates of these parameters for BTV-4, as indicated by the bimodal marginal posterior densities (Figure 5.7), which it averages out the other parameters in the model to allow us to draw conclusions about an individual parameter, and the wide credible confidence intervals for the parameters (Table 5.3). The model shows a reasonable fit to the data with the majority of observations lying within the 95% credible limits for the model predictions (Figures 5.3-5.6).

Table 5.3: Summary statistics (mean, median and 95% credible limits (CL)) for the marginal posterior densities for virus replication parameters for two strains of bluetongue virus.
† these parameters are common to both strains

parameter	BTV-1 GIB 2007/1				BTV-4 SPA 2004/02			
	mean	median	95% CL		mean	median	95% CL	
			lower	upper			lower	upper
virus titre								
maximum titre (κ)	6.76	6.76	6.56	6.98	6.28	6.28	6.07	6.51
threshold temperature (T_{min})	10.65	10.66	10.15	11.05	13.58	13.91	10.76	14.30
replication rate (α)	0.048	0.048	0.038	0.059	0.092	0.096	0.027	0.139
intial titre (mean)†	1.56	1.55	1.33	1.83	-	-	-	-
initial titre (variance)†	0.24	0.17	0.06	0.87	-	-	-	-
C_t values								
minimum C_t value (κ)	10.57	10.77	6.39	13.57	9.75	10.29	1.53	14.30
threshold temperature (T_{min})	11.37	11.32	10.55	13.18	11.99	11.81	10.78	13.70
replication rate (α)	0.0047	0.0044	0.0016	0.0117	0.0025	0.0021	0.0002	0.0072
initial C_t value (mean)†	28.60	28.60	27.56	29.67	-	-	-	-
initial C_t value (variance)†	4.54	4.12	1.78	9.65	-	-	-	-

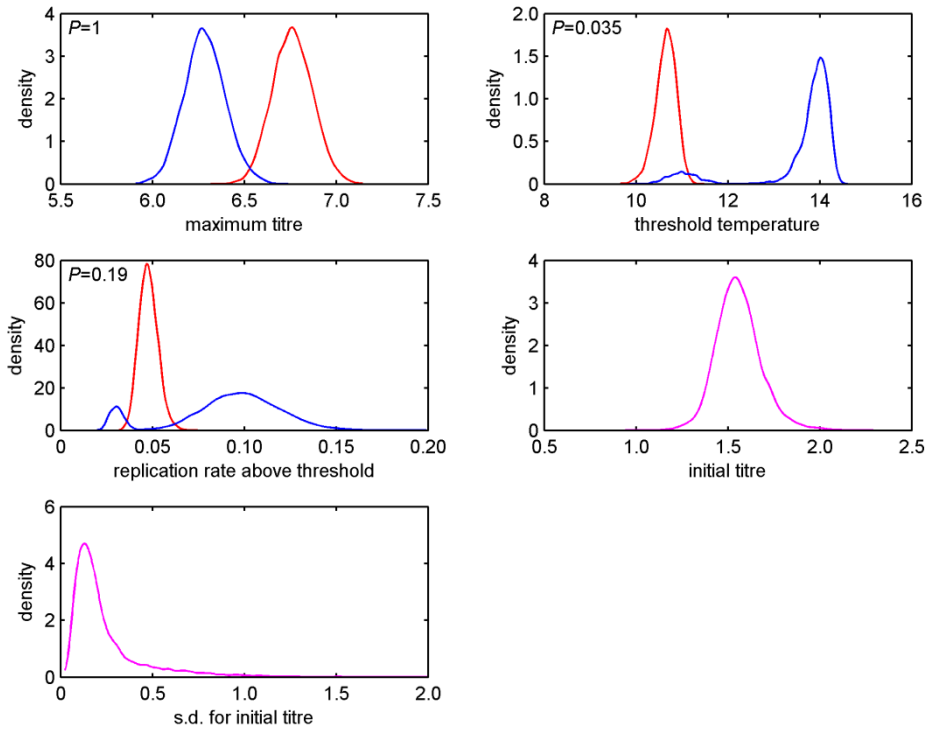

Figure 5.7: Marginal posterior densities for the parameters in the model for virus titres.
Results show the densities for BTV-1 GIB 2007/1 (red lines), BTV-4 SPA 2004/02 (blue lines) and those which are independent of strain (magenta lines). The *P* values give the probability that the parameter for BTV-1 is greater than the parameter for BTV-4 (so that *P* values in either tail (i.e. $P<0.025$ or $P>0.975$) indicate significant differences between the strains).

The same mathematical model was also used to examine variations in the viral RNA load (C_t) between the two strains. The results indicate that there were no significant differences between the strains in either the threshold temperature for replication or the virus replication rate above the threshold (Figure 5.8 and Table 5.3). The model shows a reasonable fit to the data with most observations lying within the 95% credible limits for the model predictions (Figures 5.3-5.6).

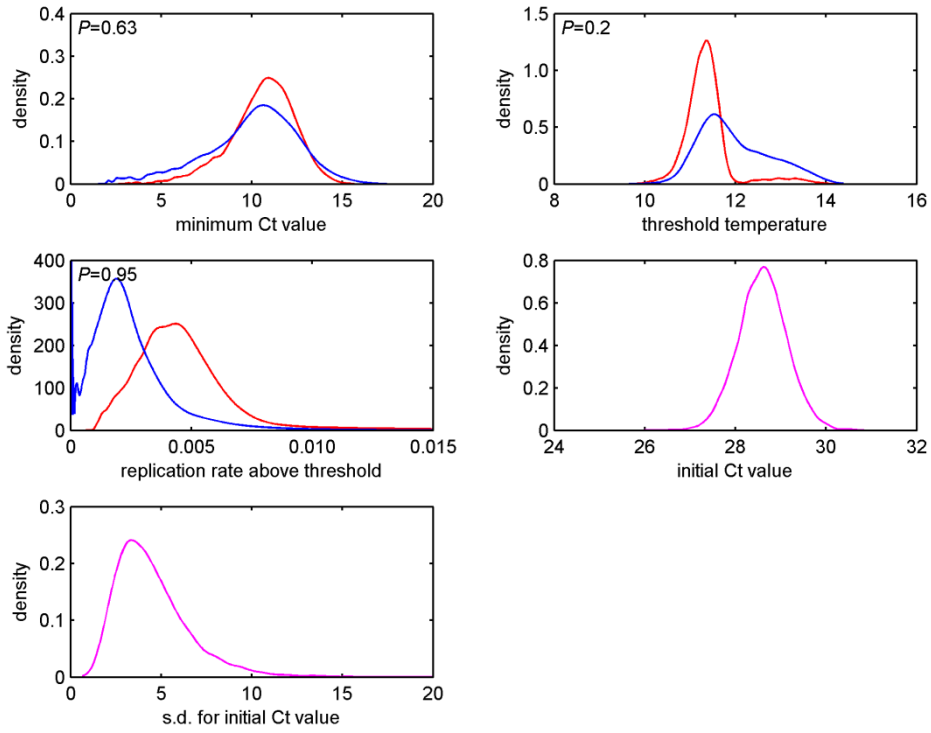

Figure 5.8: Marginal posterior densities for the parameters in the model for C_t values. Results show the densities for BTV-1 GIB 2007/1 (red lines), BTV-4 SPA 2004/02 (blue lines) and those which are independent of strain (magenta lines). The *P* values give the probability that the parameter for BTV-1 is greater than the parameter for BTV-4 (so that *P* values in either tail (i.e. $P<0.025$ or $P>0.975$) indicate significant differences between the strains).

General observations on amino acid changes within the polymerase protein VP1 of BTV strains with ability to replicate at 12°C (BTV-1 GIB2007/01), compared to (BTV-4 SPA2004/04), which did not replicate efficiently at this temperature, were carried out by consulting the dsRNA virus collection or the Pirbright Laboratory (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm). It was evinced that six amino acids had changed between BTV-1 GIB2007/01 and BTV-4 SPA2004/02, in the N-terminal and the C-terminal domains (see Table 5.4).

Table 5.4: Description of the amino acid (aa) changes and their position in VP1 between BTV-4 SPA2004/02w and BTV-1 GIB2007/01w.

	BTV-4 SPA2004/02w	**BTV-1 GIB2007/01w**
aa position	**aa**	**aa**
55	**R** (Arginine)	**Q** (Glutamine)
179	**N** (Asparagine)	**D** (Aspartic acid)
409	**A** (Alanine)	**V** (Valine)
556	**K** (Lysine)	**R** (Arginine)
1217	**S** (Serine)	**T** (Threonine)
1254	**I** (Isoleucine)	**M** (Methionine)

5.3.3 Replication of two selected BTV strains in C. sonorensis

Although BTV1w (GIB2007/01) did not replicate to a detectable level in orally-infected adult *C sonorensis*, during the first 25 days of incubation at 15ºC , there was some evidence (on days 25 and 30 p.i.) for RNA replication by BTV-4w (SPA2004/02), but no increase was detected in infectivity (Table 5.5). The first pool of orally infected *C. sonorensis* (incubated at 25ºC) showing a significant increase in BTV-1 infectivity and RNA levels (5.0 $\log_{10}$TCID$_{50}$/ml – C_t of 20.3), was detected 6 days p.i., while for the BTV-4 infected midges, first replication was detected 5 days p.i. (4.5 $\log_{10}$TCID$_{50}$/ml – C_t of 24.15). Replicating BTV was not detected, however, in pools from days 7 and 9 post-infection for BTV-1, and for days 6, 7 and 10 p.i. for BTV-4, highlighting variations in the results. A clear correlation was detected for BTV-1, between infectivity ($\log_{10}$TCID$_{50}$/ml) and viral RNA, with C_t values ranged between 20.3-19.5 at 6 and 8 days p.i.. However the BTV-4 infected insects showed less correlation between virus titre and C_t values, with C_t ranged between 24.15 to 25.72 at day 5, 8 and 9 p.i. representing between 4.5 and 1.5 $\log_{10}$TCID$_{50}$/ml.

Table 5.5: BTV replication at incubation temperatures of 15 and 25ºC in orally infected adult female *C. sonorensis*.
† days post infection* $log_{10}TCID_{50}$/ml; ** C_t

	BTV-1 GIB2007/01				BTV-4 SPA2004/02			
	15°C		25°C		15°C		25°C	
d.p.i†	Virus titre*	Viral RNA**	Virus titre*	Viral RNA**	Virus titre*	Viral RNA**	Virus titre*	Viral RNA**
0	2.25	32.94	2.25	30.92	2.5	32.78	2.25	32.78
1	-	-	2.25	29.7	-	-	≤0.5	27.25
2	-	-	≤0.5	34.2	-	-	≤0.5	29.2
3	-	-	≤0.5	no Ct	-	-	≤0.5	35.6
4	-	-	0.75	30.7	-	-	≤0.5	36.67
5	≤0.5	32.2	≤0.5	no Ct	≤0.5	31.99	4.5	24.15
6	-	-	5	20.3	-	-	≤0.5	37.33
7	-	-	≤0.5	35.7	-	-	≤0.5	no Ct
8	-	-	4.75	19.5	-	-	1.5	25.72
9	-	-	n/a	n/a	-	-	1.5	26.11
10	≤0.5	no Ct	n/a	21.83	≤0.5	no Ct	≤0.5	31.93
15	≤0.5	no Ct	-	-	≤0.5	no Ct	-	-
20	≤0.5	no Ct	-	-	≤0.5	no Ct	-	-
25	≤0.5	no Ct	-	-	≤0.5	34.95	-	-
30	-	-	-		≤0.5	28.69	-	-

5.3.4 Infection and dissemination rates for pools of C. imicola *orally infected with BTV, at 15 and 25ºC*

Pools of adult *C. imicola* incubated at 15ºC, did not show any evidence of amplification of viral RNA to 30 days p.i.. At 25ºC, amplification of BTV-1 RNA was detected on day 8 p.i., and BTV4w RNA was detected on days 5, 7 and 8 p.i., although the levels on days 7 and 8 were lower than on day 5 (Table 5.6).

Table 5.6: Replication of two BTV strains at 25ºC in orally infected adult female *C. imicola.*

Day post-infection	BTV-1w (GIB2007/01)	BTV-4w (SPA 2004/02)
	Viral RNA (C_t)	Viral RNA (C_t)
0	37.55	36.43
1	no C_t	31.55
2	no C_t	no C_t
3	no C_t	no C_t
4	no C_t	no C_t
5	no C_t	24.18
6	no C_t	no C_t
7	no C_t	31.24
8	24.21	37.36

5.4 Discussion

The primary objective of the study described in this Chapter was to produce a rapid, standardized assay that could be used to characterize and differentiate the thermal requirements for replication of different BTV strains. This was successfully achieved by inoculating BTV strains onto a *C. sonorensis* cell line (KC cells) and then measuring the quantity of infectious virus or viral RNA at selected time points, post-inoculation.

Using these techniques, it was possible to rapidly differentiate strains that could replicate under cooler conditions and hence those that might be more likely to be transmitted in northern Europe. The lower limit for BTV RNA synthesis and therefore genome amplification was previously considered to be 12-15°C (as determined *in vitro* for the reference strain of BTV-1w from South Africa (Vandijk & Huismans, 1982) and through incubation in field collected *C.imicola* (Paweska *et al.*, 2002) and *C. sonorensis* from Pirbright colony (PIRB-s-3 strain) (Wittmann *et al.*, 2002). However in the current study, three strains (BTV-1 GIB2007/01; BTV-2 SAD2002/02 and BTV-9 BOS2002/02) were found to replicate efficiently in KC cells at lower temperatures (with thermal replication limits between 10 and 12°C). Interestingly, these strains did not include the BTV-8 strain involved in northern European outbreaks (2006 to 2009),

suggesting that temperature tolerance was not a key factor in promoting transmission as appears to be the case in WNV transmission in the USA (Kilpatrick *et al.*, 2008).

More detailed analysis of BTV-1w GIB2007/01 and BTV-4w SPA2004/02 demonstrated a convincing difference in both threshold temperature for detection of replicating infectious virus (BTV-1<BTV-4), and infectious virus production above this threshold (BTV-1>BTV-4). In contrast, the results of the viral RNA load (C_t values measured by rtRT-PCR) for the two strains, showed no differences in either the threshold temperature for replication or the virus replication rate above the threshold. A possible explanation of this discrepancy could be the difficulty in the assembling viral RNA into infectious virus particles, due to variation in the temperature.

Detection of replication at lower temperatures (12°C) was consistent for BTV-1 (GIB2007/01) in both assays (virus titration and rtRT-PCR). In contrast, different patterns were observed for BTV-4 SPA2004/02 incubated at 12°C. This strain showed large variations in the data, not only between the samples collected on a daily basis but also between the two assays. Both the titration on KC cells followed by AgELISA and the rtRT-PCR, seem to be more stable for BTV-1 GIB2007/01 rather than for BTV-4 SPA2004/02. It therefore seems that the reliability of these tests in analyzing virus replication efficiency at lower temperatures could be in part dependent on the virus strain tested.

This is an important consideration to be taken into account in the use of these tests for future investigation of the effect of temperature on other BTV strains, for a better understanding of the epidemiology of BTV in northern Europe. The tests developed here were used to analyse the replication of a BTV strain that is more efficient at lower temperatures (BTV-1 GIB2007/01) and comparisons with a less-efficient strains (BTV-4 SPA2004/02). Further studies to generate more sensitive tests

for investigation of polymerase function are necessary to determine its effects on infectivity as well as RNA synthesis of other BTV strains.

Despite clear evidence for the replication of BTV-1w and BTV-4w in KC cells at 15°C, oral infection of *Culicoides* (*C. sonorensis* and *C. imicola*) did not result in significant amplification of infectious virus and only a small increase in RNA synthesis (28.69 C_t) was detected for BTV-4w at day 30 in *C. sonorensis* incubated at 15ºC, which could be due to several different reasons. Although infection may occur at lower temperatures, the rate of replication could simply be too slow to allow penetration of the midgut gut cells prior to excretion or the formation of the peritrophic matrix. As KC cells grow in multiple layers, it is also possible that they provide a larger number of cells compared to the limited numbers that are available for initial infection within the insect mid-gut. This could lead to much higher numbers of virus particles produced immediately, as a result of the initial infection allowing incubation in these cells cultures to enhance differences between the strains tested.

This study also analyses the replication efficiency of virus strains, in *Culicoides* vectors that are not present in the original areas where those BTV strains are circulating. In this study the replication efficiency of European strains of BTV were examined in a laboratory-reared North American species of *Culicoides* (*C. sonorensis*) as well as wild caught *C. imicola*, originated from South Africa. Although a larger study would provide more evidence of virus replication at different temperatures in these species, and would be needed to confirm effective transmission, virus replication was detected in both species, suggesting that the 'episystem' theory may need to be revised (Tabachnick, 2004). The use of more temperature tolerant species of *Culicoides* for these studies may allow these results to eventually be ground-truthed in the vectors, however, this will require development of more efficient feeding /oral infection methods.

In conclusion, the studies described are the first demonstrating the use of a cell based assay to detect differences in polymerase activity for BTV when incubated at different temperatures. The results in this Chapter,, suggest that the essential requirement for BTV circulation is adaptation of the virus to the local vector population and the replication rate in the vector, rather than a genetic adaptation of the virus to different temperatures.

Chapter 6: Adaptation of bluetongue virus to model systems

Full genome sequence analysis of BTV infected blood after its adaptation on mammalian (BHK-21) or insect (KC *C.sonorensis*) cells is described. The possible implication of nucleotide and amino acid changes is also discussed, together with observation of variation in infectivity of the samples according to their passage history through blood, KC *C. sonorensis* cells or BHK-21 cells. Analysis of variations in the open reading frame of the structural (VP7) and non-structural (NS2) proteins from a re-assortant strain of BTV after passage through *C. sonorensis* by oral infection, is described. The use of *Drosophila* melanogaster as a model for BTV replication studies is also investigated for the first time and its replication rate compare with the one observed in the competent vector *C. sonorensis*.

6.1 Introduction

Isolation and passage of BTV on mammalian or insects cell lines is a commonly employed technique used in both detection and propagation of strains for research. In addition, BTV passage in BHK-21 cells has long formed the basis of live attenuated vaccines worldwide with an additional step of plaque selection for non-pathogenic strains (Roy *et al.*, 2009). RNA viruses, including BTV, exist in nature as a heterogeneous population of viruses containing closely related genomes (termed a quasispecies) (Domingo *et al.*, 2012; Eigen, 1993). In addition, BTV possess a high mutation capacity, leading to the constant creation of novel variant forms with altered physiological characteristics (Domingo, 1997; Domingo & Holland, 1997; Domingo *et al.*, 1997; Domingo *et al.*, 1978; Drake & Holland, 1999; Holland *et al.*, 1982).

As discussed in Chapters 4 and 5, selection to enable replication to high titres and visible cytopathic effect in BHK-21 cell lines was traditionally a commonly used means of producing malleable strains of BTV for laboratory studies. This process could result in changes in the consensus sequence produced for a specific strain through selection of specific quasispecies and hence divergence from field populations in proteins, RNA function and ultimately phenotypic characteristics (Domingo *et al.*, 1997; Jerzak *et al.*, 2005; Schuffenecker *et al.*, 2006; Vazeille *et al.*, 2007).

Several major logistical challenges exist, however, in using purely unadapted infectious material (most commonly blood from a viraemic host) for vector competence studies, particularly in laboratories outside of endemic areas. Firstly, it is often impossible to have access to a sufficiently large volume of infectious material to perform multiple, comparable and reproducible studies. Secondly, although the sample might originate from an animal with severe clinical signs, infectivity levels may be low and may not be sufficient to infect vectors. This issue is exacerbated by the fact that commonly used long-term preservation techniques for BTV in blood frequently reduce the titre of the samples (e.g. freezing at -80°C commonly results in 1 $\log_{10}$ $TCID_{50}$ reduction in infectious titre) or involve the use of additives toxic to *Culicoides*. Thirdly, the timing of animal experiments with populations of indigenous *Culicoides* vectors is extremely difficult due to day to day variation in population abundance and activity.

Several studies have investigated the significance of genetic changes in mosquito transmitted arboviruses, and their effect on virus replication (Ciota *et al.*, 2007a; Ciota *et al.*, 2007b; Novella *et al.*, 1999; Weaver *et al.*, 1999). Alternated passages of BTV through vectors and hosts have previously shown variations in the consensus sequence of two viral proteins (VP2 and NS3/NS3A) demonstrating for the first time the founder effects caused by bottleneck in the vectors (Bonneau *et al.*, 2001).

Other studies on BTV virulent strains after attenuation in non-host cells (embryonated hens eggs ECE) have shown changes in some viral proteins (VP2 and VP5) which could result in altered cell binding properties and/or tissue tropism (Huismans & Howell, 1973). With the advent of full genome sequencing, an opportunity exists to investigate genome changes due to selection in cell culture in greater detail than previously possible. This would enable both interpretations of previous studies that utilised these strains and provide a stronger basis for validating future studies.

A second key factor in reproducing field conditions in laboratory studies of BTV transmission by *Culicoides* lies in the choice of vector species. As discussed in Chapter 1, very few species possess the required life history traits to be suitable for use in controlled experiments and there is a lack of colonised lines for the *Culicoides* genus as a whole. This has led to an almost exclusive reliance upon *C. sonorensis* as a model vector for BTV transmission without any detailed understanding of the implications of selection of what are essentially exotic strains to this species. Again, full genome sequencing allows a more detailed examination of possible changes in genotype than has been historically possible and provides the basis for comparative studies with more relevant endemic vector species.

Finally, no resources currently exist for genomic studies of *Culicoides* restricting detailed study of the mechanisms involved in BTV-*Culicoides* interactions. In contrast, the genome of the fruit fly (*Drosophila melanogaster*) has been successfully sequenced providing a vast amount of knowledge concerning viral infection and many known and characterised genetic variants are available (Cherry & Silverman, 2006; Hughes *et al.*, 2012). Infection of *D. melanogaster* with BTV has never previously been attempted, although it is known that the majority of arthropods will at least partially replicate arboviruses if they are directly inoculated into the haemocoel. Demonstration

of replication of BTV in this species would, if carefully characterised, provide a new model to examine pathways of immune response that would complement future studies of the *C. sonorensis* genome.

The purpose of this chapter is therefore to examine:

1) Changes in the BTV genome following adaptation to replication in cell culture and potential implications for virus phenotype.
2) Changes in the genome of BTV following passage through *C. sonorensis*.
3) *Drosophila melanogaster* as a potential model species for the study of BTV infection and dissemination.

6.2 Material and Methods

6.2.1 Analysis of infectivity and full genome sequence variation of BTV-8 UKG2007/06 after adaptation to mammalian (BHK-21) and insect (KC) cell lines.

Infectious bovine blood containing BTV-8 (sample UKG2007/06 collected in EDTA), from the index case for the first BTV-8 outbreak in the UK during 2007 (http://www.reoviridae.org/dsRNA_virus_proteins/outbreaks.htm#BTV8-UKG-2007) (Chapter 2, Table 2.1) was used for these experiments. The bovine blood sample was washed and sonicated (Chapter 4, Section 2.1.1) before being used to generate strains of differing degrees of adaptation to cell culture. Following an initial passage on the KC cells (*C. sonorensis* cell line), strains were then passaged a further four times on the KC cells and one and five times on a BHK-21 cell line. This generated strains of the following designation: KC_1; KC_5; KC_1BHK_1; KC_1BHK_5 (Chapter 2, Section 2.1.1 and

2.1.2). Supernatants from infected cells layers were collected following methods described in Chapter 2 section 2.1.1 for BHK-21 cells and 2.1.2 for KC infected cells.

The infectivity of supernatants from infected cell layers were measured by titration on BHK-21 cells or by titration on KC cells followed by transfer to BHK-21 cells (Chapter 2, Section 2.4.2 Figure 2.5). Viral RNA from infectious blood and cell supernatant was extracted using a 96 well Universal BioRobot® (Qiagen) and tested by rtRT-PCR (Chapter 2, Section 2.5.1 and 2.5.2). The nucleotide sequence of purified BTV RNA from the infectious blood sample was determined using an Applied Biosystems 3730 BigDye ddNTP capillary sequencer, (Maan *et al.*, 2010), while the sequencing of purified RNA from the passaged virus samples was carried out at the Welcome Trust Sanger Institute (UK), as part of a collaboration with IAH, using a 454 second generation sequencer (Caporale *et al.*, 2011). All analyses of the consensus sequences for the BTV segments were performed at IAH, using SeqMan software (DNAStar Inc.) (Maan *et al.*, 2010), by Dr Kyriaki Nomikou, a senior Postdoc within the Molecular Reference Research Group at the Pirbright laboratory, as part of a collaborative study.

6.2.2 Analysis of infectivity and nucleotide changes in BTV-4 MOR2009/09 after oral infection of C. sonorensis

A Morocco field isolate of BTV-4 (MOR2009/09) was used for this experiment (see http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-4.htm#MOR2009/09) (Chapter 2, Table 2.1). This virus is a reassortant between parental lineages, represented by BTV-4 (MOR2004/02) and BTV-1 (MOR 2006/06) (Table 6.1). These two parental strains are phylogenetically identical (100% nucleotide similarity) to BTV-2SPA2004/02 and BTV-1GIB2007/01 described in Chapter 5 of

this manuscript. The peculiarity of these two strains is that they have demonstrated to have different polymerase activity at lower temperatures (12C). Hence, the choice of a strain (BTV-4MOR2009/09) that is a re-assortant between the two parental strains BTV-2SPA2004/02 and BTV-1GIB2007/01was here used to investigate variations in the genome sequence of segment-7 and segment-10 after passage through *Culicoides* vectors.

Table 6.1: Genetic analyses of the BTV-4 strain (MOR2009/09)
Nucleotide sequence identity levels are shown between the 'progeny strain' BTV-4 (MOR2009/09) (which circulated in the Mediterranean region during 2007-2009) and its parental strains of BTV-1 and BTV-4 (represented by MOR2004/02 and SPA2004/02, or MOR2006/06 and GIB2007/01 respectively).
In circle the two segments that were here investigated (Segment-7 and Segment-10)

BTV-1 parental strain 1MOR2006/06, 1GIB2007/01 nt % similarity with BTV-4 daughter strain	BTV-4 daughter strain 4MOR2009/09 Genome segment Number	BTV-4 parental strain 4MOR2004/02, 4SPA2004/02 nt % similarity with BTV-4 daughter strain
99.8% →	1	98.8%
50.9%	2 ←	99.6%
94.9%	3 ←	99.3%
99.8% →	4	95.9%
100% →	5	96.8%
68.9%	6 ←	99.6%
100% →	(7)	94.1%
99.7% →	8	95.8%
97.3%	9 ←	99.4%
99.5% →	(10)	98%

Two to three days old *Culicoides sonorensis* (n=~250), were fed (Chapter 2, Section 2.2.1) with the reassortant BTV-4 strain (MOR2009/09) which had been passaged two times on KC cells (KC_2) (Chapter 2, Section 2.1.2). The infectivity of the inoculum and the homogenates (Chapter 2, Section 2.3) of orally infected midges (day 0 and day 7 p.i.), was determined by titration on KC cells followed by AgELISA (Chapter

2, Section 2.4.2 and Chapter 4, Section 4.2.1). Viral RNA was extracted using a QIAmp® Viral RNA Mini Kit (see Chapter 2, Section 2.5.1) and analysed by rt-RT-PCR (Chapter 2, Section 2.5.2).

Sequencing of the viral RNA and analysis of the consensus sequence data were performed in collaboration with Dr Kyriaki Nomikou from IAH Pirbright (Section 6.2.3.1). The sequences of Seg-10 (NS3/NS3A) and Seg-7 (VP7) of the virus in each individual insect homogenate were compared against the inoculum (BTV-4 MOR2009/09) to determine nucleotide identity levels and identify amino acid changes.

6.2.3 Investigation of BTV replication in Drosophila melanogaster: the use of Drosophila as a tool to explore the genetic basis of virus-vector interactions

BTV-1 GIB2007/01 KC_2 (passaged twice in the KC cell line) was used to study the replication of BTV in IT inoculated *D. melanogaster*. In parallel a sample of the same virus (GIB2007/01) was also passaged once to KC_2 to generate isolate GIB2007/06, the full genome of which has been sequenced. Adult female *D. melanogaster* were supplied by Dr. Frederick Arnaud from the University of Lyon, as part of a collaborative study. Two to three days old adult female *C. sonorensis* (n=200) and three to five days old *D. melanogaster* (n=500) were intra-thoracically injected with 0.2µl and 0.345µl respectively of a BTV-1 (GIB2007/01 - KC_2) virus suspension (Chapter 2, Section 2.2.3). A further 137 *D. melanogaster* were also injected with uninfected Schneider's medium as a negative control. The *C. sonorensis* were incubated at 25°C, fed daily on a wet cotton pad (5% sucrose solution) and at day 3, 7 and 10 p.i. five insects were collected for analysis.

The adult *D. melanogaster* were also incubated at 25°C, inside plastic conical flasks and maintained on a standard corn-meal-agar medium (David, 1962). In this case

two parallel experiments were run at the same time, using separate conical flasks, containing 146 and 147 insects respectively. A total of 10 insects (5 from each flask) were collected individually on a daily basis up to day 10 p.i., except for day 0 where 20 females (10 from each flask) were collected. The mock injected *Drosophila* were tested at day 0, 7 and 10 post infection.

Individuals of both species were homogenised in 1ml of Schneider's medium (Chapter 3 and Chapter 2, Section 2.3), then titrated on the KC cell line (Chapter 2, Section 2.4.2) followed by AgELISA (Chapter 4, Section 4.2.1). BTV RNA was extracted from a sample of the homogenate (50 µl) using the Universal BioRobot® (Qiagen, crawley, UK) then assessed by rtRT-PCR (see Chapter 2 Section 2.5.1 and 2.5.2). Statistical analyses were performed by Dr Christophe Terzian from the University of Lyon. Median values for virus replication data ($log_{10}TCID_{50}$/ml or C_t values) were analysed by 'one-way analysis of variance by rank', Kruskall-Wallis and pair-wise Wilcoxon tests (Petrie & Watson, 2006).

6.3 Results

6.3.1 Analysis of infectivity and sequence variations in BTV-8 UKG2007/06 after adaptation to mammalian (BHK-21) and insect (KC) cell lines

6.3.1.1 Infectivity, viral RNA and infection rate analysis

The direct titration of BTV-8 (UKG2007/06) infected blood did not result in CPE in BHK-21 cells, despite the presence of significant amounts of viral dsRNA in the inoculum (Table 6.2). Similar results were also obtained for titration of the KC_1 passaged strain on BHK-21 cells with no CPE again observed. When the same two samples (blood and KC_1 passage) were subsequently titrated and incubated on KC cells for 7 days at 25ºC, then transferred to BHK-21, CPE was observed, recording a virus

titre of 2.5 and 2.0 $log_{10}TCID_{50}$/ml respectively. The KC_5 passage showed a virus titre of 2.5 log_{10} $TCID_{50}$/ml when titrated directly on BHK-21 cells and 3.0 $log_{10}TCID_{50}$/ml in KC cells (when transferred to BHK cells).

Following the first passage on BHK-21 cells (KC_1BHK_1), the virus caused clear CPE, with a titre of 5.25 log_{10} $TCID_{50}$/ml when titrated on BHK cells and 6.25 log_{10} $TCID_{50}$/ml in KC cells (by transfer to BHK cells) (Table 6.2). Four further passages on BHK cells (KC_1BHK_5) recorded only a small further increase of 0.5 log_{10} $TCID_{50}$/ml compared to the first BHK passage and of 0.75 log_{10} $TCID_{50}$/ml in KC cells, when measured by transfer.

The level of viral RNA detected by rtRT-PCR was lowest in the original blood sample (27.68 C_t), but the C_t values decreased substantially following a single passage in the KC cell line (KC_1 = 20.22 C_t). After four further passages in KC cells (KC_5) the C_t value had decreased further to 10.82 C_t (Table 6.2). The C_t value of the first passage in KC cells (at 20.22 C_t) fell further to 14.42 on the first pass in the BHK-21 cell line, and still further to 11.83 C_t by the fifth passage.

Table 6.2: Viral RNA measured by rtRT-PCR (C_t value) and infectivity ($log_{10}TCID_{50}$/ml) of infectious BTV-8 blood (UKG2007/06) and its further passage on KC and BHK cells

Passage history	rtRT-PCR (C_t values)	Virus titre in BHK-21 cells $log_{10}TCID_{50}$/ml	Virus titre in KC cells, followed by transfer to BHK-21 cells $log_{10}TCID_{50}$/ml
Blood	27.68	no CPE	2.5
KC_1	20.22	no CPE	2
KC_5	10.82	2.5	3.0
KC_1BHK_1	14.42	5.25	6.25
KC_1BHK_5	11.83	5.75	5.5

6.3.1.2 Full genome sequence analysis

Full genome sequence analyses were carried out on the blood and all of the cell passages (KC_1, KC_5, KC_1BHK_1, KC_1BHK_5). Amplification and sequencing of the full length of Seg-6 from the blood was not possible, due to degeneration of the sample caused by its storage at -80ºC. Moreover, for Seg-1, Seg-3 and Seg-8 (from the blood as well) only 56%, 36% and 98% of the nucleotides were sequenced and amplified due to the same reason).

Analysis of the changes in the nucleotide sequence, between the original BTV strain in the blood sample and the 4 subsequently produced strains are given in Tables 6.4 to 6.7. No nucleotide changes were recorded in Seg-7 and Seg-10 between any of the strains. The highest numbers of nucleotide changes were recorded in Seg-1 and Seg-3 between the virus from the blood and all other cell passages (52-53 nucleotide changes in Seg-1 and 22 nucleotide changes in Seg-3) (Table 6.3). No other changes were recorded in Seg-1 during passage in KC cells (KC_1 to KC_5) but seven nucleotide changes were detected in Seg-1 of KC_1BHK_1 compared to KC_1. When KC_1BHK_1 was passaged four times in BHK cells (KC_1BHK_5), two more nucleotide changes (position

74 and 878) were detected in Seg-1. One nucleotide, at position 2753 in Seg-3, had also changed from KC_1BHK_1 to KC_1BHK_5.

One passage of the virus on KC cells (KC_1) resulted in 41 nucleotide changes in Seg-4, which were then maintained throughout all of the other passage levels tested. Seg-5 of the same passage (KC_1) showed a deletion in the 5'NCR (at position 9) but once the sample had been passaged 5 times on KC cells (KC_5), or in BHK cells once to five times ($KC_1BHK_1$and KC_1BHK_5), this deletion was no longer present. Only KC_1BHK_5 had differences in the nucleotide sequence of Seg-6, with one deletion in the 5'NCR (pos. 9) and 1 nucleotide change in 5'NCR (pos. 18). After one passage of the blood in KC cells (KC_1), one nucleotide had changed in Seg-8 (position 1102), which was then maintained throughout the remaining four passages on KC cells (KC_5). There was also a further nucleotide change in position 19 in this final passage. When KC_1 was passaged on BHK cells (KC_1BHK_1), a nucleotide change that occurred in Seg-8 between KC_1 and the blood (position 1102), was maintained (Table 6.4). However, after 5 more passages on BHK cells (KC_1BHK_5), two new nucleotide changes were recorded in Seg-8 (positions 13, 1102 and 1120).

Analysis of amino acids examining changes between virus in the original blood sample and all four of the cell culture grown viruses, demonstrated little variation. Six amino acid changes in positions 94,168,169,243,343, and 371 were recorded in segment four of the KC_1 passage, compared to the VP4 sequence from the original blood sample. These changes were maintained throughout all of the passages, both in KC (KC_5) or BHK cells (KC_1BHK_1 and KC_1BHK_5). The only other amino acid change observed, was in VP2 of the KC_5 passage virus, with a change at position 390 to a polar uncharged neutral (isoleucine), instead of the neutral nonpolar serine. This change was only detected in KC_5 but not in any of the other passage levels.

Table 6.3: Comparison of nucleotide and amino acid changes between the BTV-8 UKG2007/06 field strain and four strains subsequently generated by passage.

BTV-8 (UKG2007/06)	Nucleotide changes	Region (% sequenced)	Amino Acid changes
KC1	53	S-1 (56%)	-
KC5	53		
KC1BHK1	52		
KC1BHK5	53		
KC1	-	S-2 (100%)	1 (390)
KC5	1		
KC1BHK1	-		
KC1BHK5	-		
KC1	22	S-3 (36%)	-
KC5	22		
KC1BHK1	22		
KC1BHK5	22		
KC1	41	S-4 (100%)	6 (94,168,169,243,343,371)
KC5	41		6 (94,168,169,243,343,371)
KC1BHK1	41		6 (94,168,169,243,343,371)
KC1BHK5	41		6 (94,168,169,243,343,371)
KC1	Deletion 5'NCR position 9	S-5 (100%)	-
KC5	-		
KC1BHK1	-		
KC1BHK5	-		
KC1	-	S-7 (100%)	-
KC5	-		
KC1BHK1	-		
KC1BHK5	-		
KC1	1	S-8 (98%)	-
KC5	2		
KC1BHK1	-		
KC1BHK5	-		
KC1	Deletion 5'NCR position 9	S-9 (100%)	-
KC5	Nucleotide mixed base 17 (G/T)		
KC1BHK1	Nucleotide mixed base 17 (G/T)		
KC1BHK5	Nucleotide mixed base in pos. 17 (G/T)		
KC1	-	S-10 (100%)	-
KC5	-		
KC1BHK1	-		
KC1BHK5	-		

Table 6.4: Comparison of the nucleotide/amino acid changes between BTV-8 UKG2007/06 KC_1 passage and 3 comparative strains subsequently generated by passage.

BTV-8 (UKG2007/06)	Nucleotide changes (position)	Region	Amino Acid changes
KC5	-		-
KC1BHK1	7 (1385, 2216, 2228, 2240, 2249, 2252, 2285)	S-1	-
KC1BHK5	1 (74)		-
KC5	1 (1186)		1 (390)
KC1BHK1	-	S-2	-
KC1BHK5	-		-
KC5	-		-
KC1BHK1	-	S-3	-
KC1BHK5	1 (2753)		-
KC5	-		-
KC1BHK1	-	S-4	-
KC1BHK5	-		-
KC5	-		-
KC1BHK1	-	S-5	-
KC1BHK5	-		-
KC5	-		-
KC1BHK1	-	S-6	-
KC1BHK5	Deletion in 5'NCR(9) and 5'NCR (18)		-
KC5	-		-
KC1BHK1	-	S-7	-
KC1BHK5	-		-
KC5	3 (14, 19, 1122)	S-8	-
KC1BHK1	1 (1102)		-
KC1BHK5	3 (13, 1102, 1120)		-
KC5	1 mixed base in 17 (G/T)	S-9	-
KC1BHK1	1 mixed base (17) (G/T) and 1 change (30)		-
KC1BHK5	1 mixed base in 17 (G/T)		-
KC5	-	S-10	-
KC1BHK1	-		-
KC1BHK5	-		-

Table 6.5: Comparison of the nucleotide/amino acid changes between BTV-8 UKG2007/06 KC_5 passage and two comparative cell passages.

BTV-8 (UKG2007/06)	Nucleotide changes (position)	Region	Amino Acid changes
KC1BHK1	7 (1385, 2216, 2228, 2240, 2249, 2252, 2285)	S-1	-
KC1BHK5	2 (74, 878)		-
KC1BHK1	1 (1186)	S-2	1 (390)
KC1BHK5	1 (1186)		1 (390)
KC1BHK1	-	S-3	-
KC1BHK5	1 (2753)		-
KC1BHK1	-	S-4	-
KC1BHK5	-		-
KC1BHK1	-	S-5	-
KC1BHK5	-		-
KC1BHK1	-	S-6	-
KC1BHK5	Deletion in 5'NCR(9) and 5'NCR(18)		-
KC1BHK1	-	S-7	-
KC1BHK5	-		-
KC1BHK1	4 (14, 19, 1102, 1122)	S-8	-
KC1BHK5	4 (14, 19, 1102, 1122)		-
KC1BHK1	1 (30)	S-9	-
KC1BHK5	-		-
KC1BHK1	-	S-10	-
KC1BHK5	-		-

Table 6.6: Comparison of the nucleotide/amino acid changes between BTV-8 UKG2007/06 KC_1 BHK_1 passage and KC_1 BHK_5.

Passage history	Nucleotide changes (position)	Region	amino acid changes
KC1BHK5	2 (74, 878)	S-1	-
KC1BHK5	-	S-2	-
KC1BHK5	1 (2753)	S-3	-
KC1BHK5	-	S-4	-
KC1BHK5	-	S-5	-
KC1BHK5	1 deletion in 5'NCR(9) and 1 in 5'NCR (18)	S-6	-
KC1BHK5	-	S-7	-
KC1BHK5	2 (13, 1120)	S-8	-
KC1BHK5	1 (30)	S-9	-
KC1BHK5	-	S-10	-

6.3.2 Analysis of infectivity and nt/amino acid changes in BTV-4 MOR2009/09 after oral infection and passage through C. sonorensis

6.3.2.1 Infectivity, viral RNA and infection rate in C. sonorensis *orally infected with BTV-4 (MOR2009/09)*

The virus titre of the BTV-4 reassortant (MOR2009/09) was 4.5 $log_{10}TCID_{50}$/ml by titration on KC cells (followed by AgELISA) (Chapter 2, Section 2.4.2 and Chapter 4, Section 4.2.1). Amplification on KC cells was therefore used to increase its infectivity prior to feeding *C. sonorensis*. Infectious titre following passage was measured as 6.75 $log_{10}TCID_{50}$/ml and the strain was then diluted to give a final concentration of 6.0 $log_{10}TCID_{50}$/ml in the blood mixture (diluted 1:2) to feed *C. sonorensis.*

The virus titre of orally-infected adult female *C. sonorensis* that were individually homogenized immediately after the blood meal (Day 0 p.i.), ranged from 2.5 to 3.0 $log_{10}TCID_{50}$/ml. After 7 days incubation, a total of 13 insects were positive (52%), eight of which (32%) possessed replicating virus (titre between 2.5-4.75 $log_{10}TCID_{50}$/ml) and C_t values of between 23.54 and 30.04. Thirteen insects (52%) were

negative for BTV in both of the assays (rtRT-PCR and virus titration). Two insects (numbers 11 and 23), which possessed high infectivity levels (3.75 and 4.75 $log_{10}TCID_{50}$/ml respectively) and C_t values of 27.50 and 26.94 respectively, were chosen for the virus-genome sequence analysis.

6.3.2.2 Analysis of genome sequence forSeg-7 and Seg-10: comparison among the reassortant strain, parental strains and oral fed infected C. sonorensis

6.3.2.2.1 Seg-7 / VP7 sequence variations

Nucleotide analysis of the Seg-7 open reading frame (Table 6.7) recorded a similarity of ≥ 99.7% between BTV-4 MOR2009/09 (the reassortant progeny strain) and the virus from the two infected insects (insect 11 and insect 23) (Table 6.7 'a' and 'b').

Table 6.7: Nucleotide changes in Seg-7 of reassortant BTV-4 MOR2009/09 (inoculum) after passage in orally infected *C. sonorensis*

a)	**Female n.11**		
	Inoculum/*Culicoides*	**BTV-1 MOR06/06/ *Culicoides***	**BTV-4 MOR2004/02/ *Culicoides***
	Seg-7 (18-1064)	**Seg-7 (18-1064)**	**Seg-7 (18-1064)**
Similarity	99.7%	99.7%	93.8%
Changes	9A insertion 41A-42G 492A-493G 900C-901T 1148T-1148 deletion	9A insertion 41A-42G 492A-493G 900C-901T 1148T-1148 deletion	9A insertion 71 nt changes 1148 deletion
b)	**Female n.23**		
	Inoculum/*Culicoides*	**BTV-1 MOR06/06/ *Culicoides***	**BTV-4 MOR2004/02/ *Culicoides***
	Seg-7 (18-1064)	**Seg-7 (18-1064)**	**Seg-7 (18-1064)**
Similarity	99.7%	99.7%	93.8%
Changes	A41G A492G C900T	A41G A492G C900T	71 nt changes

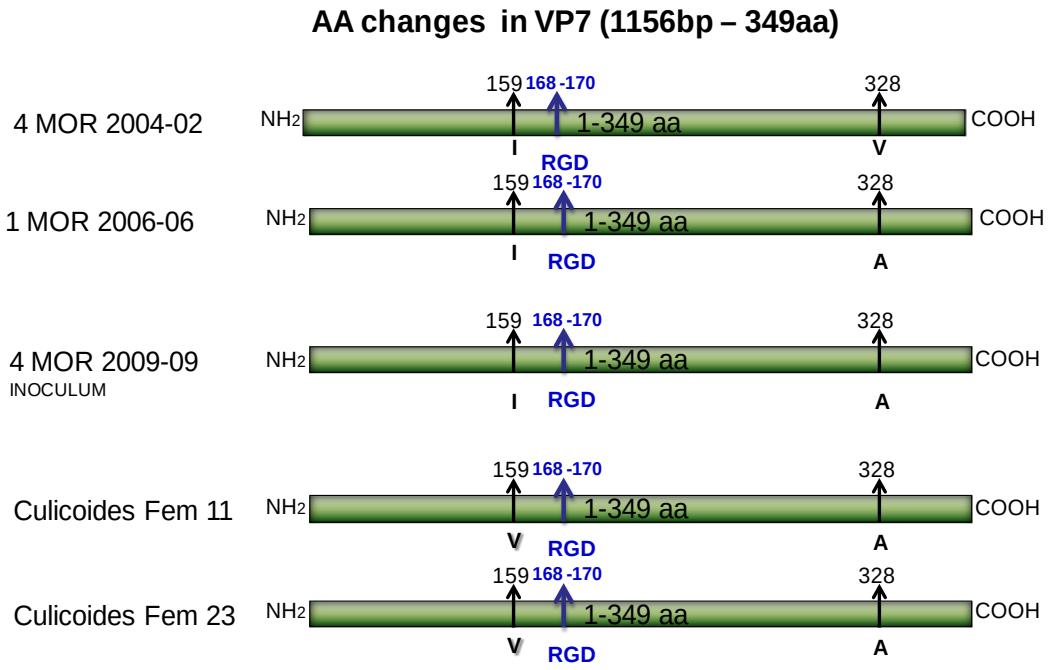

Figure 6.1: Amino acid changes in VP7 between the reassortant BTV-4 (MOR2009/09), its parental strains and after oral infection of *C. sonorensis*.

NH2 Amino terminal; COOH Carboxyl terminal; I: Isoleucine – nonpolar, neutral; A: Alanine - nonpolar, neutral; V: Valine - nonpolar, neutral; K: Lysine – polar, positive; R: Arginine – polar, positive; T: Threonine – polar, neutral; E: Glutamic acid – polar negative

Virus from both *Culicoides* homogenates showed two non-polar amino acid changes in VP7: one in position 159 (isoleucine (I) →valine (V), both non-polar amino acid) and one in position 328 (alanine (A) instead of valine (V)) (Figure 6.1). The change at position 159 was not observed in the BTV-4 reassortant strain, or either of the two parental strains. No other amino acid differences were observed (Figure 6.1). No variation was observed in the RGD (putative integrin-binding motif) of VP7 in any of the samples. Mutations in this tripeptide region of Seg-7 have previously been shown to reduce the binding efficiency of core particles to *Culicoides* cells (Tan *et al.*, 2001).

6.3.2.2.2 Seg-10 / NS3/NS3A sequence variations

Table 6.8: Similarities and nucleotide changes in Seg-10 of reassortant BTV-4 MOR2009/09 (inoculum) after passage in orally fed *C. sonorensis* (female n.11).

	Female n.11		
	Inoculum/ *Culicoides*	**BTV-1 MOR06/06/ *Culicoides***	**BTV-4 MOR2004/02/ *Culicoides***
Similarity	**Seg-10 (20-706)** 99.5%	**Seg-10 (20-706)** 99.1%	**Seg-10 (20-706)** 97.8%
Changes	G235A A322G G592A C598T R803A Y804T R808A R810A	A235A A322G A576G A592A C598T A722G A751G A773G A803A T804T G808A F8010A T812T	G178A T220A C290T G298A A322G A334G A520G C598T C634T G659A G664A T670A A711G G721A T784C G808A G810A T812C

More changes in nucleotide and amino acid were observed in Seg-10 and NS3/NS3A proteins (Table 6.8, 6.10 and Figure 6.2), with nucleotide identity levels ranging between 97.8 to 99.5% (see Table 6.8). No variations in either nucleotide or amino acid were observed in Seg-10 between the two orally infected insects (insect 11 and 23).

Table 6.9: Nucleotide changes in Seg-10 of reassortant BTV-4 MOR2009/09 (inoculum) after passage in orally fed *C. sonorensis*.

b)	**Female n.23**		
	Inoculum/ *Culicoides*	**BTV-1 MOR06/06/ *Culicoides***	**BTV-4 MOR2004/02/ *Culicoides***
	Seg-10 (20-706)	**Seg-10 (20-706)**	**Seg-10 (20-706)**
Similarity	99.5%	99.3%	97.9%
Changes	G235A	A235A	G178A
	A322G	A322G	T220A
	G592A	A576G	C290T
	C598T	A592A	G298A
	R803A	C598T	A322G
	Y804T	A722G	A334G
	R810A	A751G	A520G
		A773G	C598T
		T804T	G659A
		G808R	G664A
		G810A	T670A
		T812T	A711G
			G721A
			T784C
			G808R
			G810A
			T812C

All of the amino acid changes recorded were in the intracellular domain (183-229 amino acid) of NS3/NS3A (Figure 6.2). A substitution in position 214 from the non-polar amino acid alanine (present in the parental strain of BTV-4 (MOR2004/02)), to the polar amino acid threonine, was observed in both of the two infected insects. Two other amino acid changes were also observed (in NS3/NS3A) between the two infected insects and the BTV-1 parental strain (MOR2006/06): at position 225 (glutamic (E) instead of Lysine (K)), and at position 186 (arginine instead of lysine from the parental strain).

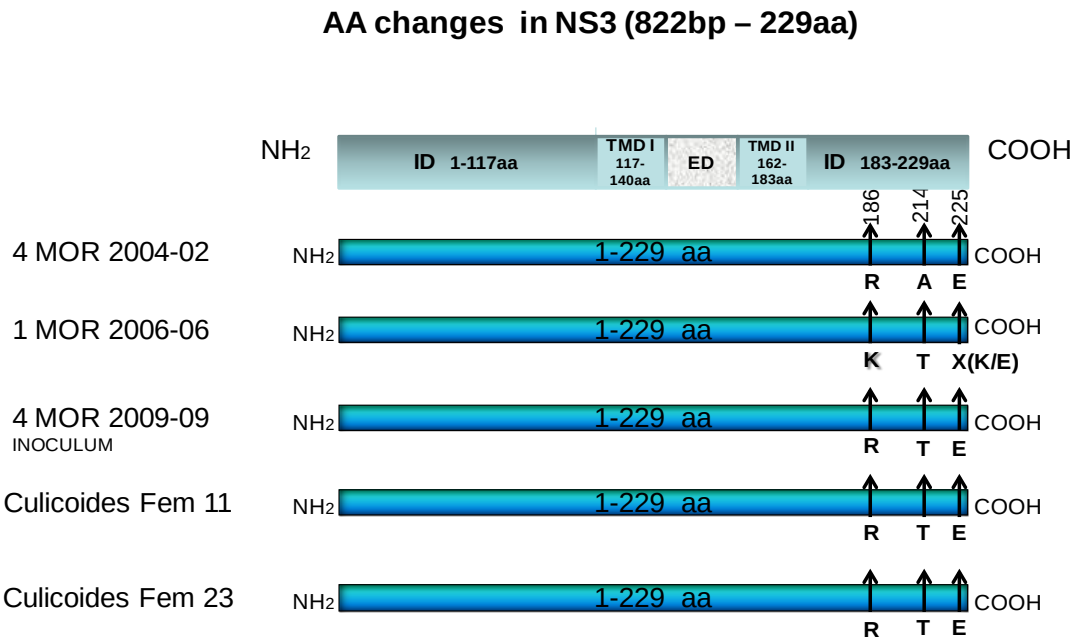

Figure 6.2: Amino acid changes in NS3/NS3A between BTV-4 (MOR 2004/02), BTV-1 (MOR 2006/06) and BTV-4 (MOR2009/09) (before and after passage in orally infected *C. sonorensis*). NH2 Amino terminal; COOH Carboxyl terminal; ID Intracellular domain; TMD I transmembrane domain I; TMD II Transmembrane domain II; ED Extracellular domain; I: Isoleucine – nonpolar, neutral; A: Alanine - nonpolar, neutral; V: Valine - nonpolar, neutral; K: Lysine – polar, positive; R: Arginine – polar, positive; T: Threonine – polar, neutral; E: Glutamic acid – polar negative

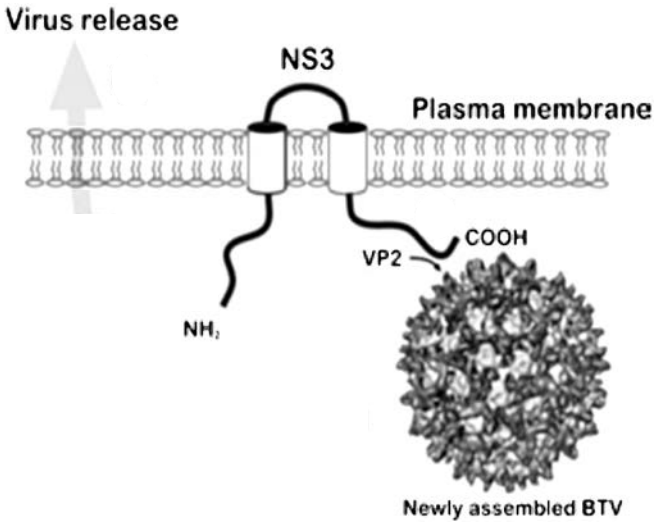

Figure 6.3: NS3/NS3A BTV protein and its position in cellular membrane.

Modification of a picture from: Beaton A.R. et al. 2002. ©2002 by The National Academy of Sciences

6.3.3 BTV replication in Drosophila melanogaster

6.3.3.1 Measurement of viral RNA and infectivity in intrathoracically infected C. sonorensis *and D. melanogaster*

The survival rate of *D. melanogaster* at 10 days post intrathoracic inoculation with BTV-1 GIB2007/01 KC_2 passage, was 78.77%, with 75.91% survival of the mock infected insects. The virus titre recorded in each insect is represented for *D. melanogaster* and *C. sonorensis* in Figure 6.4. Significant differences were observed in the median values for virus titres in infected *Drosophila* at days 3 post infection (p.i.) comparing to day 0 p.i. (Figure 6.5), confirming the initiation of virus replication. One-way analysis of variance by rank indicated that the median of titration values ($log_{10}TCID_{50}$/ml) varied significantly at day 0, day 3 and day 10 post infection (Kruskall-Wallis test, $P < 10^{-6}$: pair-wise Wilcoxon tests, $P<0.01$). This confirmed not only that amplification of BTV started by day 3 p.i. (one adult *D. melanogaster* out of 10 contained 4.0 $log_{10}TCID_{50}$/ml and a mean C_t value of 22.94 by rtRT-PCR), but also an increase of virus replication reaching a peak by day 8 to 10 p.i. (with 4.75 and 5.0 $log_{10}TCID_{50}$/ml and a mean of 21.92 and 20.88 C_t values respectively) (Figure 6.4, 6.7). The number of *D. melanogaster* that were completely negative for infectious virus particles by using both the two techniques, are given in Table 6.10.

At day 0 post infection, the virus titre detected in infected *C. sonorensis* was between 1 to 2.5 $log_{10}TCID_{50}$/ml (Figure 6.4), with mean C_t values ranging between 29.45 to 31.78, while at day 3 post infection, titres of 4.5 to 5.5 $log_{10}TCID_{50}$/ml and mean C_t values of 17.57 to 21.4 were observed among the 5 infected insects (Figure 6.4 and 6.7). Infectivity in the intrathoracically infected *C. sonorensis* recorded a peak in virus titre at day 7 post infection, with 6.0 $log_{10}TCID_{50}$/ml in 4 out of 5 insects and 5.75

in one insect (figure 6.4), with a mean C_t value ranging between 15.55 and 17.36 (Figure 6.7).

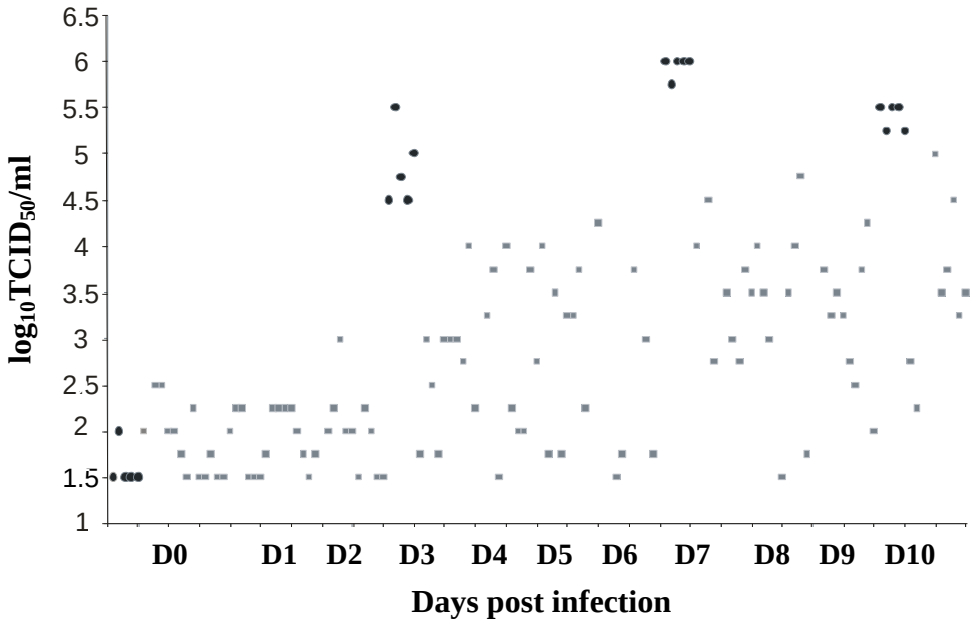

Figure 6.4: Infectious virus particle ($log_{10}TCID_{50}$/ml) detected in each individual *D. melanogaster* (gray squares) and *C. sonorensis* (black squares) infected with BTV-1 GIB2007/01 KC_2 at daily interval post intrathoracic inoculation.

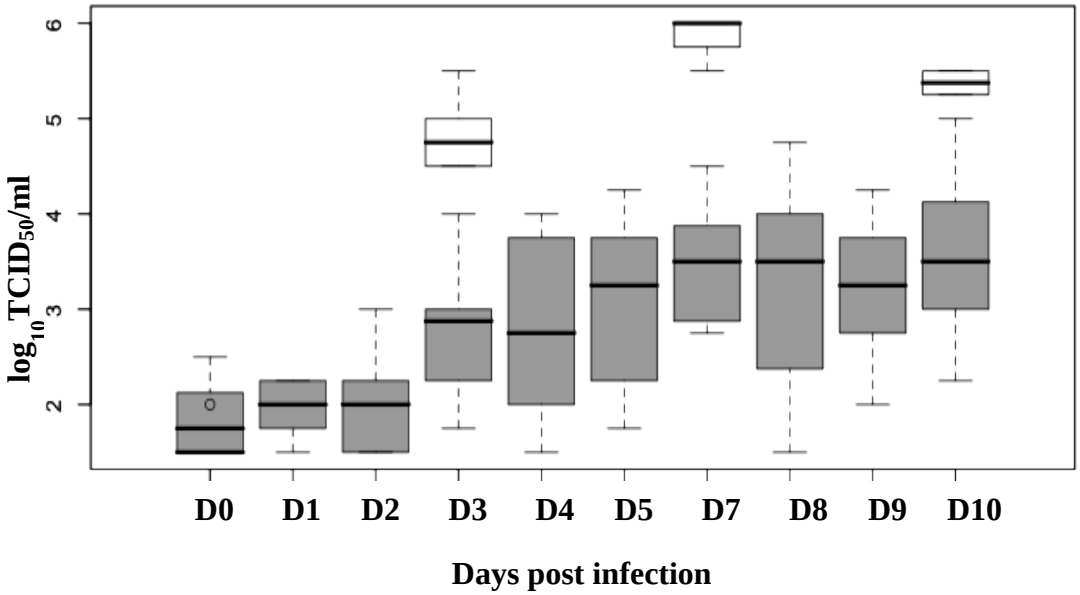

Figure 6.5: Boxplot of virus titre ($log_{10}TCID_{50}$/ml) from *D. melanogaster (*gray boxes) and *C. sonorensis* (white boxes) infected with BTV-1 GIB2007/01 KC_2 at daily interval post intrathoracic inoculation.

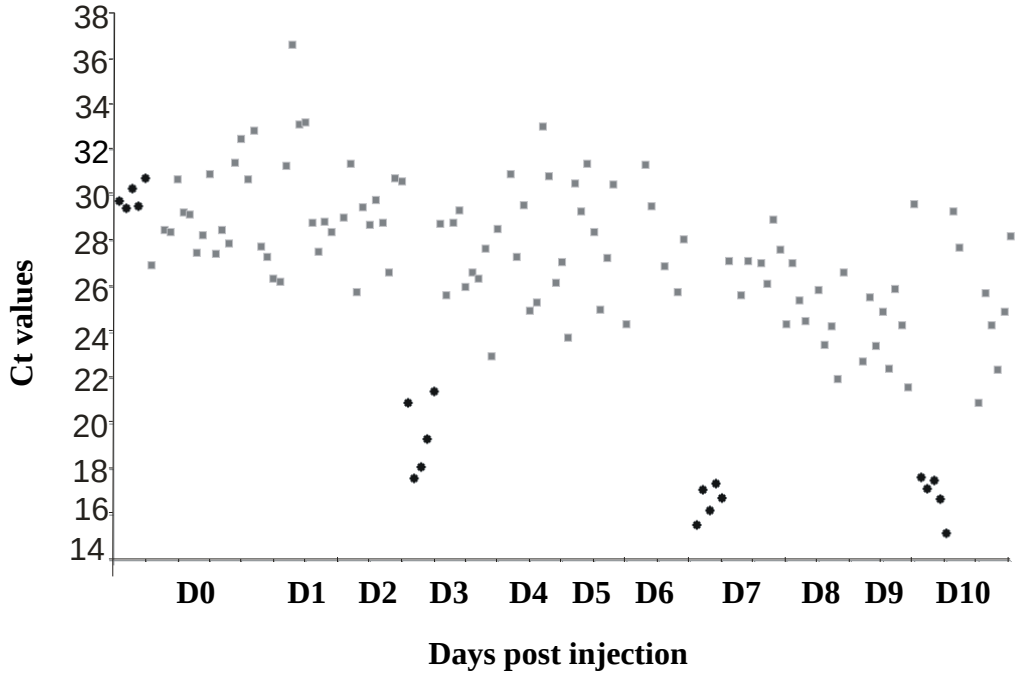

Figure 6.6: rtRT-PCR (C_t) data recorded from each individual *D. melanogaster* (gray squares) and *C. sonorensis* (black squares) infected with BTV-1 GIB2007/01 KC_2 at daily interval post intrathoracic inoculation.

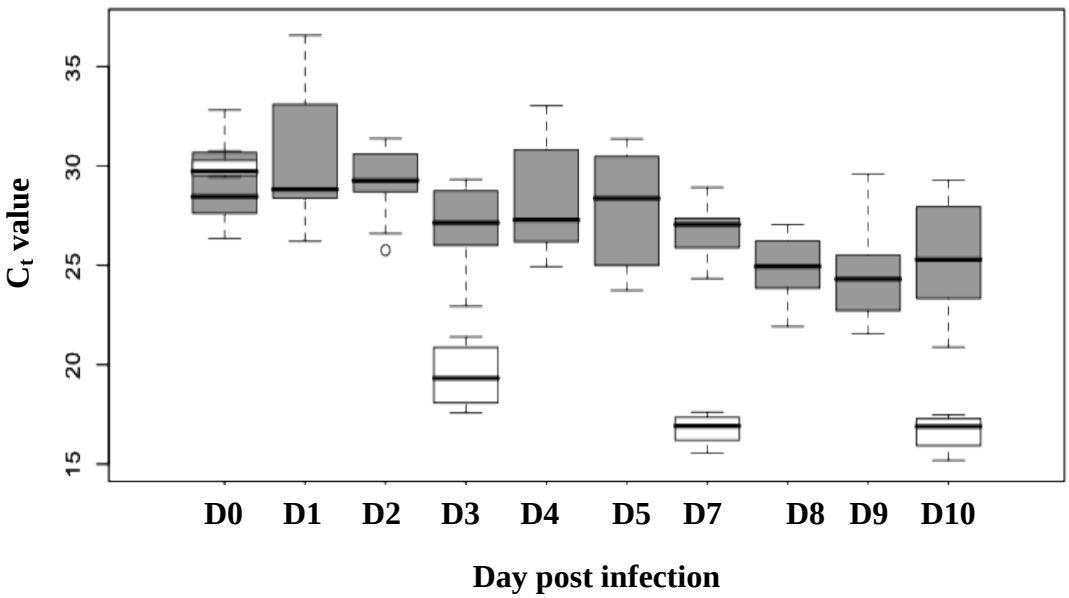

Figure 6.7: Boxplot of viral RNA (C_t) from *D. melanogaster (*gray boxes) and *C. sonorensis* (white boxes) infected with BTV-1 GIB2007/01 KC_2 at daily interval post intrathoracic inoculation.

Table 6.10: The number of *D. melanogaster* that were negative by virus isolation on KC cells ($log_{10}TCID_{50}$/ml) and rtRT-PCR, at each day post intrathoracic infection with BTV-1 GIB2007/01 KC_2.

	Days post infection										
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Total number of *Drosophila* tested / day	20	10	10	10	10	10	10	10	10	10	10
Number of insects negative by virus titration and rtRT-PCR	5	1	4	0	1	2	6	2	2	1	2

The highest number of negative *D. melanogaster* was recorded at day 6 p.i. (6 out of 10) (Table 6.10). The individual values obtained by virus titration and rtRT-PCR are shown

in figure 6.4 and 6.6, although due to high mortality at day 6, the analysis of the boxplot (Figure 6.5 and 6.7) are not displayed, as mean values were only available for four insects.

6.4 Discussion

This chapter has examined the impact of using model systems to explore BTV infection and replication in cell culture and *C. sonorensis* adults and additionally explored a novel methodology involving the infection of *D. melanogaster*. The considerable advances in sequencing technology made in this field enable these preliminary studies to demonstrate that changes in the genome of BTV occur following adaptation to both cell lines and vector *Culicoides*. In addition, the definition of a novel system for investigating BTV infection pathways in *D. melanogaster* demonstrated significant promise for study of molecular pathways (albeit with reservations concerning direct relevance to the field as *D. melanogaster* is not haematophagous and hence does not require blood meal).

As expected, the original BTV-8 (UKG2007/06) field strain underwent greatest population selection during replication on the KC cell line. This was characterised by relatively low levels of RNA replication in the initial KC cell line passage (constituting a decrease in C_t value from 27.68 to 20.22), multiple amino acid changes in genome segment four (seven changes recorded: a divergence of 1.1% of total amino acids) already after one passage on KC cells and a single amino acid change in genome segment two (a divergence of 0.1%) only after 5 passages in KC cells. While the functional significance of these changes was not investigated in detail during this study and the full extent of changes across the genome could not be assessed due to a failure in amplification from several segments of the original infected blood sample

(highlighting an issue with extraction efficiency from samples), divergence from the original field strain in a large proportion of the genome was clearly extremely restricted.

Following the initial genomic bottleneck imposed by isolation on cell culture, there were very few subsequent changes during passage in either KC or BHK-21 cell lines. After the initial passage of the blood into KC cells (KC_1), 41 nt changes were observed but the first and only amino acid change during subsequent passages on KC cells was recorded only after 4 consequents passages of the blood on KC cells. 1Following passage in the KC cell line the BTV-8 strain was subsequently able to infect BHK cells efficiently with a virus titre of 5.25 $\log_{10}TCID_{50}$/ml (C_t = 14.42) and induced cytopathic effects (CPE). Interestingly, this selection was accompanied by very few amino acid changes across the genome, all of which were silent in resulting amino acids. The positions of nucleotide changes that did occur in segment one (encoding VP1: the viral polymerase) were mostly localised (position 1385, 2216, 2228, 2240, 2249, 2252 and 2285) although the significance of this remains unclear.

Repeated passage in KC *C. sonorensis* and BHK-21 cells similarly also had little effect on the genome of the BTV-8 strain, although selection was evident from the limited increase in viral RNA following initial inoculation from the KC_1 strain to the KC_1BHK_1. While subsequent passage of these strains in their respective lines led, as expected, to increases in titre visible by CPE (in the case of the BHK_5 strain) and viral RNA detected, again, only one amino acid change was noted between KC_1 and KC_5 and no amino acid changes were found between KC_1BHK_1 and KC_1BHK_5. While this again implies that selection of strains following inoculation into cell cultures is extremely limited, the functional role of segment two, where the change occurred in KC_5, is of interest. VP2 is centrally involved in cell-attachment and penetration by intact BTV (Hassan & Roy, 1999; Mertens *et al.*, 1989) and is hence involved in cell-entry (and

possibly also exit). Hence, changes in this protein could potentially affect infectivity in specific cell types.

Interestingly, neither genome segment ten (encoding NS3 and NS3a) nor seven (encoding VP7) showed any variation from the field strain in any of the passaged strains. Both of these segments have been implicated as being involved in cell entry and exit although exact mechanisms remain unclear. There is evidence that NS3/NS3a (encoded by Seg-10) is involved in cell exit (Guirakhoo *et al.*, 1995) (Hyatt *et al.*, 1993; Owens *et al.*, 2004) and this hypothesis is also supported by the fact that in the BHK-21 cell line NS3/NS3a is generated in small amounts and may be largely absent in the late stage of infection, when the CPE is starting (Hyatt *et al.*, 1993). Segment seven forms the outer surface of the BTV core particle and is therefore involved in the infectivity of BTV cores for insect cells (Grimes *et al.*, 1995). Although BTV core-particles have a reduced infectivity for mammalian cells, they are highly infectious for insect cells, suggesting that the virus can use a different mechanism of virus entry in insect cells and in the adult vector, which does not involve VP2 or VP5 (Mertens *et al.*, 1996; Xu *et al.*, 1997).

A key consideration in the interpretation of results of this chapter is the method employed to produce the full genome sequences. Traditional sequencing techniques derive a consensus sequence that is not representative of quasispecies diversity within each sample. Hence, the sequence derived is likely to represent the most common strain among an unknown diversity of multiple closely related strains. It is therefore impossible to judge whether the process of selection results in a reduction in overall strain quasispecies diversity or if these are retained in samples following passage. An obvious method to examine this question would be through next generation sequencing assays (Caporale *et al.*, 2011; Jere *et al.*, 2011) which provide measures of within

sample diversity. This would additionally allow separation between selective events and novel mutations in treated samples.

While taking this limitation into account, the fact that such little variation was observed in consensus sequences across the strains examined appears to indicate that the impact of selection/mutation was minimal in the primary quasispecies within strains produced. The changes in segment 4 following passage of the field strain into the KC cell line, represents only a very small part of the BTV genome still they are of significant interest, and could be investigated by generation of strains by reverse genetics (Boyce & Roy, 2007; Matsuo *et al.*, 2010). This would allow these changes to be assessed functionally in a variety of cell lines or hosts. Sequence analysis of the virus genome after virus replication in *C. sonorensis*, in particular of NS3/NS3a and VP7, and possibly after alternation using a live system between natural hosts and vectors (Bonneau *et al.*, 2001) would also contribute to a better understanding of variation among alternated passage of BTV in different cell lines.

Comparison of the sequences of BTV segment seven and segment ten of a reassortant BTV-4 MOR2009/09 strain, before and after amplification in orally infected *C. sonorensi,s* also generated few apparent differences in consensus sequence. No amino acid differences were present in VP7 or VP10 between the two orally infected adult *Culicoides* but one amino acid differences was observed between the inoculum (BTV-4 MOR2009/09) and both the two *Culicoides* (female 11 and 23). A second amino acid change was also observed between one of the two parental strains (BTV-4 MOR2004/02) with the inoculum. In Seg-10, no amino acid changes were observed between the inoculum and both the two *Culicoides* but, at least one amino acid change has been recorded between the inoculum (BTV-4 MOR2009/09) and the parental

strain BTV-1 MOR2007/06 form which Seg-10 derive from. The functional properties of these changes have not been investigated and could form a part of future work.

A major consideration in this study was providing a detailed history of the strain concerned, allowing retrospective characterisation of phenotypes for each strain. The BTV-4 MOR2009/09 reassortant strain has shown a non-synonymous substitution in position 328 of VP7 comparing to its parental strain BTV-4 MOR2004/02 with alanine instead of valine of the BTV-4 MOR2004/02. The other parental strain (BTV-1 MOR2006-06) presented the same amino acid of the reassortant BTV-4 MOR2009/09. This is explained by the derivation of VP7 of the reassortant BTV-4 MOR2009/09 from the other parental strain BTV-1 MOR2006/06 (100% nucleotide sequence similarity). Preliminary data generated in Chapter 5 suggest that the parental strains of this reassortant have different phenotype characteristics when incubated at lower temperatures (12ºC). Future studies are therefore suggested looking at the variability in other proteins of this and additional BTV reassortants to correlate them with their vector competence observed in *Culicoides* species.

In the final section of this Chapter, the replication of BTV in *Drosophila melanogaster* was demonstrated for the first time after injection via an intrathoracic route. Despite *D. melanogaster* not being a haematophagous insect, it has obvious utility as a model organism as evidenced by the vast body of literature describing its biology (Hoffmann, 2003; Rubin, 1988). This discovery could open a new field of virus-vector interaction studies, exploiting the unique understanding of innate immunity available for *D. melanogaster* which is already being utilised in studies of mosquito-borne pathogens (Chotkowski *et al.*, 2008). Given the ease of rearing and maintaining *D. melanogaster* lines, this should also open up novel areas of research to the virological community that have to date been limited by availability of *Culicoides*

colony lines. In the event of a *Culicoides* genome being generated they will also provide a powerful comparative system for analysis. It is clear, however, that these studies would require links to increase the number and quality of *Culicoides* colonies to retain an applied value in the field. In conclusion, the data presented demonstrate that the vast majority of adaptation of an infectious BTV blood sample on mammalian or insect cells occurs in the first isolation of the virus. Even during this process, a maximum of only 53 nucleotide changes were observed at different segments of the full genome while only few amino acid changes were recorded, in Seg-2 and Seg-4. Comparison of nucleotide and amino acid changes of BTV reassortant strain after passage in *C. sonorensis* shown higher number of changes in NS2 protein comparing to VP7 and no differences were observed for both the two proteins among the two orally infected *C. sonorensis* individuals. This chapter also successfully demonstrated for the first time the amplification of BTV in intrathoracically infected *Drosophila melanogaster*, reaching a peak virus titre of 5.0 $\log_{10}TCID_{50}$/ml after 10 days incubation at 25ºC. This has the potential to provide a new alternative model for studying immunological responses to BTV infection.

Chapter 7: General discussion, conclusions and future directions

A clear understanding of virus- and vector-related factors influencing BTV distribution within Europe is of paramount importance in our understanding the epidemiology of the disease and in predicting and preventing its spread. A key issue in collecting and generating useful data to accomplish this aim, lies in producing reproducible and standardized assays that are transferable between laboratories. Despite several publications highlighting this requirement (Batten *et al.*, 2008a; Carpenter *et al.*, 2009; Quan *et al.*, 2010), this aspiration remains far from being fulfilled. In addition, the continuous and ongoing evolution and implementation of novel detection assays places significant demands on laboratories to continually update their diagnostic technologies and methodologies.

The results described in this thesis contribute significant advances in creating a framework to investigate BTV-*Culicoides* interactions worldwide and present significant data concerning the global epidemiology of the virus. The development of an optimised and standardised Tissue Lyser based assay (Kato & Mayer, 2007) to quantify infectious BTV (and BTV RNA) in *C. sonorensis* samples removes uncertainty regarding the impact and consistency of homogenisation that had yet not been considered in these studies (Chapter 3). It also provides a basis for reproducible processing of individual *Culicoides* in a high throughput assay, achieved (for the first time) using a piece of equipment that is already present in a significant number of diagnostic laboratories (Chisenhall & Mores, 2009; Joest *et al.*, 2010; Richards *et al.*, 2009).

Frontline detection technologies for BTV, in particular real-time RT-PCR (rtRT-PCR), were investigated for detection of BTV in *Culicoides* and offer significant advantages over more traditional virus isolation techniques in terms of sensitivity, reproducibility, throughput and robustness (Chapters 4 & 5). When paired with quantification of BTV using the KC cell line (derived from *C. sonorensis*) and an AgELISA developed during the study, this provides a powerful set of tools to investigate replication of the virus in *Culicoides* and in the KC cell-line itself. It was also demonstrated that rtRT-PCR assays could be used to discriminate transmissible infections. As rtRT-PCR is now the most commonly used assay in 'frontline' diagnostics for BTV in Europe (Shaw *et al.*, 2007) and several other regions worldwide, this validation represents significant progress towards a coherent framework for competence testing.

The value of this system can be seen in the attempts to implicate specific vector species for BTV-8 in northern Europe (from 2006-2011) which were characterised by a lack of standardisation, a poor understanding of infection mechanisms and ultimately a failure to convincingly identify any single species as a vector (Becker *et al.*, 2010; Dijkstra *et al.*, 2008; Mehlhorn *et al.*, 2007; Meiswinkel *et al.*, 2007; Sabio *et al.*, 2006). The identification of standardised methods to accurately estimate infection and dissemination rates in *Culicoides,* raises the possibility of studies to fully re-assess the 'episystem hypotheses' (Gibbs & Greiner, 1994; Tabachnick, 2004) for BTV transmission and determine its validity.

In the current study northern and southern European strains of BTV were shown to replicate in both *C. sonorensis* and *C. imicola* (Chapter 4), although the vast majority of relationships between field, or low passage strains of BTV, and major vector species remain to be explored. Given the results of previous studies, any future studies should

take into account intra-species variation in vector competence between different insect populations (Carpenter *et al.*, 2006; Tabachnick, 1996a).

The use of confocal microscopy in Chapter 4 provides a means of visually investigating barriers to BTV dissemination. When paired with the recently developed reverse-genetic techniques, this has the potential to revolutionise our understanding of BTV replication in adult *Culicoides* through the generation of strains with specific phenotypes and subsequent studies of infection and dissemination. This includes the potential to generate fluorescent tagged strains that can be visibly tracked within individual adult *Culicoides* without the need for antibody-based detection (and the associated issue of cross reactions discussed in Chapter 4) (Shaw *et al.*, 2012).

While less commonly used than real-time RT-PCR worldwide, the development of a quantified isolation system based upon the KC cell line is also key in allowing assessment of infectious virus levels, without the need for pre-adaptation to less relevant cell lines. KC cells are now utilised by a range of laboratories for research concerning *Culicoides*-borne pathogens. In addition to further analyses suggested in Chapter 6 concerning changes in the genome of strains isolated from field samples, it would also be useful to consider both the impact of repeated cell passage on sensitivity and infection of the line by BTV. Previous studies on the use of this cell line for detection of BTV replication, reported CPE (as a result of infection) in early passages of the cells (Wechsler *et al.*, 1989). Hence, effects related to the age of the cells, on virus amplification should be considered. These studies could also encompass the production of novel cell lines from other vector species (particularly *C. imicola*, where it is feasible to mass produce large numbers of eggs for these studies).

In addition to providing tools for investigating *Culicoides* vector competence, this thesis has also generated significant novel data concerning the epidemiology of

BTV and factors underlying global movement of strains. The use of KC cells to rapidly screen differences in replication across BTV strains, demonstrated differences in both threshold-temperatures and replication rates (Chapter 5). While these had previously been investigated for orbiviruses (AHSV, EHDV and BTV) (Mullens *et al.*, 1995; Paweska *et al.*, 2002; Wittmann & Baylis, 2000), the validity of these earlier studies is limited by the use of viruses that had been passaged repeatedly in mammalian cell lines and could only statistically infer replication at <15°C. The demonstration that at least some strains of BTV can replicate between 10-12°C changes the paradigm used to assess onwards transmission risk in northern Europe, although this still requires confirmation in adult *Culicoides* of species that are sufficiently robust to survive under cooler incubation temperatures.

Interestingly, tolerance of cooler incubation temperatures was not a consistent indicator of BTV strains that had successfully replicated and were transmitted in northern Europe. The discovery that BTV-8 strains (even those that had been present in the region for over a year) were not able to replicate at 12°C was striking. It has been suggested that at the time of initial establishment of the virus (Summer/Autumn 2006), temperatures were exceptionally high, ranging substantially above the seasonal average (van Oldenborgh, 2007; Wilson & Mellor, 2009b). Hence, even if the genotype of these strains was not suitable for virus replication at low temperature, it would have not represented a limitation to the initial spread of the virus. This could potentially have been a key factor in the emergence of BTV-8 (Mintiens *et al.*, 2008; Wilson & Mellor, 2009b), as it is thought that this strain was introduced into the region from a sub-Saharan afro-tropical climate (Maan *et al.*, 2008), allowing no opportunity for selective adaptation to northern European conditions.

Unlike BTV-8, BTV-1 followed an incursion route of sequential movement through North Africa, Spain and France over a period of several years. While this afforded opportunities for adaptation to a cooler climate, it was evident from the analysis of this strain during the present study that tolerance was already present before the movement into northern Europe. While requiring detailed phenotypic confirmation from strains of BTV-1 isolated further north in France, this allows a comparison of genetic parameters that could be involved in the efficiency of viral polymerase activity (Vandijk & Huismans, 1982). The availability of a genome sequence database for the analysis of possible nucleotide or amino acid changes in virus strains, and their correlation with replication efficiency. has potential to contribute to this area (particularly leading from the studies of reassortant strains described in Chapter 6, or by creation of novel mono-reassortants between selected strains by 'reverse-genetics').

The impact of adaptation of BTV strains to replication in mammalian (BHK-21) or KC cells was also investigated using a BTV-8 strain (UKG2007/06) (Chapter 6). This addresses a key question underlying previous studies of BTV epidemiology, as a large proportion of historic studies relied on cell passaged strains (Luedke *et al.,* 1977; Takamatsu *et al.,* 2003; Venter *et al.,* 1998), that were sufficiently malleable for laboratory use (Kirkland & Hawkes, 2004). While the consequences of imposing selective bottlenecks appeared minor, representing changes of only a small proportion of total nucleotide or amino acid sequences, the functional impact of these changes remains unclear and should be addressed in future work, a similar conclusion to that reached concerning passage of BTV-4 MOR2009/09 through *C. sonorensis*.

The use of *Drosophila melanogaster* as a model for investigation of the role of genetic mutations in the insect and implications for control of the pathogen's replication and dissemination has already been initiated in mosquitoes (Chotkowski *et al.,* 2008).

Despite the fact that this insect is not haematophagous and not closely related to *Culicoides*, the available information regarding its genome could be used for detailed studies on mechanisms involved in virus-vectors interactions. The advantages of using this species also include straightforward rearing and maintenance due to its large size and very high survival rate following intrathoracic inoculation, all pointing towards the possibility of its use in future studies.

This study has highlighted general advances in technology (e.g. full genome sequencing for BTV and reverse genetics) that may aid our understanding of BTV epidemiology and ultimately enhance control of epidemics. A key consideration is the advent of genomics and the increasingly available sequencing capacity for vector species. To date no genomic studies have been initiated for *Culicoides*, however due to its behavioural malleability *C. sonorensis* is by far the most likely candidate in the genus for this process. This would provide not only an opportunity for comparison with other vector groups but also provide a vast area of research across many disciplines (Arensburger *et al.*, 2010; Severson & Behura, 2012; Terenius *et al.*, 2008). A primary area that has to date been unexplored for *Culicoides* is the innate immune response to infection with BTV. In combination with characterisation of endosymbionts (including *Wolbachia*), this is an area that could become a major focus of future studies with control applications (Glaser & Meola, 2010).

Other potential areas are less technologically demanding and specific but also have the potential to vastly improve our understanding of BTV. In investigations of vector competence in the field, the present study has detailed approaches that can be employed to assess dissemination in field collected individuals (Chapter 4). The recent development of honey coated FTA cards to detect arbovirus transmission by mosquitoes in the field, has the potential to enhance this area by allowing rapid assessment of virus

transmission (Hall-Mendelin *et al.*, 2010). However, this technique has not yet been validated for use with *Culicoides* and may be constrained both by the small size of vectors and the sensitivity of assay used for detection.

Another important area is ecological investigation and colonisation of additional of *Culicoides* species (other than *C. sonorensis and C. nubeculosus*). While this process has been initiated with *C. imicola* (Veronesi *et al.*, 2009), which is currently the most epidemiologically important *Culicoides* vector of BTV worldwide (Paweska *et al.*, 2002; Venter *et al.*, 2006), techniques for feeding and rearing northern Palaearctic species remain in their infancy. One reason for this is that species involved in transmission of BTV within the *C. obsoletus* and *C. pulicaris* groups have not been clearly defined (Carpenter, 2009), reducing the benefit of establishing colonies of any single species. Despite this, it is clear that *C. obsoletus* s.s. by its abundance, overlap in distribution with areas of BTV circulation (Purse *et al.*, 2005) and demonstrated vector competence, is very likely to play at least a partial role in transmission (Caracappa *et al.*, 2003; De Liberato *et al.*, 2005; Ferrari *et al.*, 2005).

While the current study has defined methods for carrying out vector competence investigations of northern Palaearctic *Culicoides* a major barrier remains in being able to elicit a consistent and comparable blood-feeding response (Venter *et al.*, 2005). Initial studies have been conducted using pledglet feeding, rather than membrane-based techniques, leading to a potential lack of comparability with other studies of *Culicoides* worldwide. Pledglet feeding does not provide a large enough blood-meal to initiate oogenesis, reducing its utility for colonisation attempts (unpublished data). Achieving the objective of a sustainable colony of northern European species would allow detailed comparative infection studies with those described in Chapter 4, with BTV strains recorded in northern Europe as well as those from other putative episystems. It is also

an essential prerequisite for studies of genomics, which have so far relied upon vectors that are reared in the laboratory only (in part due to the requirement for highly inbred lines that have a reduced microbiota load).

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Appendix 1

List of publications and conferences attended during PhD Studies

***Publications*:**

Andrew Shaw, **Eva Veronesi**, Guillemette Maurin, Najate Ftaich Francois Guiguen, Frazer Rixon, Maxime Ratinier, Peter Mertens, Simon Carpenter, Massimo Palmarini, Christophe Terzian, and Frederick Arnaud. 2012. "*Drosophila melanogaster* as a model organism for bluetongue virus replication and dissemination". Journal of Virology. September, 86 (17): 9015-9024

Veronesi E., P.P.C. Mertens, A. Shaw, P. Mellor and S. Carpenter. 2008. "Quantifying bluetongue virus in *Culicoides* biting midges (Diptera: *Ceratopogonidae*). Journal of Medical Entomology; 45(1):129-132.

Simon Carpenter, Anthony Wilson, James Barber, **Eva Veronesi**, Gert Venter and Simon Gubbins. 2011. "Temperature dependence of the extrinsic incubation period of orbiviruses in *Culicoides* biting midges". Plos One. 6(11):e27987. [Epub 2011 Nov 18].

Sushila Maan, Narender S. Maan, Kyriaki Nomikou, **Eva Veronesi**, Katarzyna Bachanek-Bankowska, Manjunatha N. Belaganahalli, Houssam Attoui and Peter P.C. Mertens. 2011. "Complete genome characterisation of a novel 26th bluetongue virus serotype from Kuwait". Plos One; 6(10) p.e26147

Maan, S., S.N Maan, N. Ross-smith, C.A. Batten, E.A. Shaw, S.J. Anthony, A.Samuel, K.E. Darpel, **E. Veronesi**, C.A.L. Oura, K. Singh, K. Nomikou, C. Potgieter, H. Attoui, E. van Rooij, P. van R.K. De Clercq, F. Vandenbussche, S. Zientara, E. Breard, C. Sailleau, M. Beer, B. Hoffman, P.S. Mellor, P.P.C. Mertens. 2008. "Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains". Virology August 1; 377 (2): 308-18.

Oral presentations

"*Culicoides* and vector competence for Schmallenberg virus (SBV)". 2012 European SOVE meeting, 8-12th of October, 2012 Montpellier (France).

"*Culicoides* and vector competence for Schmallenberg virus (SBV)". 2012 SOVE Annual conference; 23-27th of September 2012 St. Augustine, Florida (US)

"A better understanding on detection and prevention of vector-borne diseases: *Culicoides* bitin midges and the diseases they transmit" 8th September 2012 - Johns Hopkins University (Baltimore – US).

“Comparison of Bluetongue virus dissemination among two *Culicoides* vectors: *C. imicola* and *C. sonorensis*”. 2011 ESA annual meeting – Reno (US) 13-16 November 2011.

“Bluetongue virus and its emergence across Europe. Can we control vector borne diseases?” 17th of May 2011. University of Zurich (Switzerland)

"Bluetongue disease: what remains to be discovered about the virus and its interaction with the vectors (*Culicoides* biting midges)?" 28th of March 2011. Onderstepoort Veterinary Institute – Onderstepoort (South Africa).
“*Drosophila melanogaster* (Diptera: *Drosophilidae*) as an In vivo model for Bluetongue virus replication studies”. European SOVE meeting, 13-18 September 2010. Wroclaw (Poland).

Bluetongue virus and its emergence across Europe. Can we control vector borne diseases?” 23rd of June 2010. International Atomic Energy Agency (IAEA) Vienna (Austria).

“Vector competence for *C. sonorensis* using BTV different strains and temperatures” MedReoNet, 3-7 May, 2010. Tunis (Tunisia).

“Interaction between Orbiviruses and their *Culicoides* vectors”. MedReoNet, 2-4 December, 2009. Lisbon (Portugal).

"Variation in transmission competence of *Culicoides*, and the genetic basis for differences in transmissibility of BTV strains”. Society of Vector Ecology (SOVE). 11-18 October 2009. Antalya (Turkey).

“Investigation of the mechanisms involved in vector competence for orbiviruses and their interactions with vector species (biting midges)”. European Mosquitoes Control Association, 9-12 march 2009. Turin (Italy).

“Validation of a diagnostic technique for Bluetongue Virus detection and quantification in adults of *Culicoides* biting midges (Diptera: *Ceratopogonidae*)”. 2nd Annual meeting of Epizone. 4th-6th June 2008. Brescia (Italy).

“Preliminary observation on the *C. imicola* colonisation: notes on larval development at 3 temperatures under laboratory conditions” Society of Vector Ecology (SOVE 2008) conference 25th-28th of March 2008. Cambridge (UK).

“Validation of a diagnostic technique for Bluetongue Virus detection and quantification in adults of *Culicoides* biting midges

(Diptera: *Ceratopogonidae*)" WAVLD (World Association of Veterinary Laboratory Diagnosticians) Symposium –10th -14th November 2007. Melbourne (Australia).

"Validation of a diagnostic technique for Bluetongue Virus detection and quantification in adults of *Culicoides* biting midges (Diptera: *Ceratopogonidae*)" EMCA 2007 meeting. 10th – 14th September 2007. Prague (Czech Republic).

"Replication of Live attenuated bluetongue vaccine viruses in a European breed of sheep, before and after passage in vector midges (Diptera: *Ceratopogonidae*)". "2ème Journée Scientifique sur les maladies animals transfrontalieres ». 23rd of March 2006 - Gammarth (Tunisia).

***Other publications*:** Veronique Moulin, Cor Vonk Noordegraaf, Birgit Makoschey, Mirjam van der Sluijs, **Eva Veronesi**, Karin Darpel, Peter P.C. Mertens and Hans de Smit. "Clinical disease in sheep caused by bluetongue virus serotype 8 and prevention by an inactivated vaccine". Vaccine. 12/2011; 30(12):2228-35.

K.E. Darpel, P. Monaghan, J. Simpson, S. Anthony, **E. Veronesi**, H.W. Brooks, H. Elliott, J. Brownlie, H.-H. Takamatsu, P.S. Mellor and P.P.C. Mertens. 2012. "Involvement of the skin during bluetongue virus infection and replication in the ruminant host". Veterinary research. Veterinary research 2012 04/2012; 43(1):40.

E. Veronesi, Karin E. Darpel, Chris Hamblin, Simon Carpenter, Haru-Hisa Takamatsu, Simon J. Anthony, Heather Elliott, Peter P.C. Mertens, Philip S. Mellor. Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4. Vaccine 28 (2010) 1397–1403

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Karin E. Darpel, Carrie A. Batten, **Eva Veronesi**, Susanna Williamson, Peter Anderson, Mike Dennison, Stuart Clifford, Ciaran Smith, Lucy Philips, Cornelia Bidewell, Katarzyna Bachanek-Bankowska, Anna Sanders, Abid Bin-Tarif, Anthony J. Wilson, Simon Gubbins, Peter P.C. Mertens, Chris A. Oura, and Philip S. Mellor. Transplacental Transmission of Bluetongue Virus 8 in Cattle, UK. Emerging Infectious Diseases Vol. 15, No. 12, December 2009.

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Veronesi E., C. Hamblin, P.S. Mellor. 2005. "Studies on live attenuated BTV vaccines in Dorset Poll sheep before and after passage in vector midges (Diptera: *Ceratopogonidae*)" Vaccine, 23(48-49): 5509-5516

Other oral presentations:

"Replication of Live attenuated bluetongue vaccine viruses in a European breed of sheep, before and after passage in vector midges (Diptera: *Ceratopogonidae*)". 8th –11th November 2005, Virology Africa 2005 - UCT Graduate school of Business, Cape Town (South Africa).

"Live attenuated bluetongue vaccine viruses in a European breed of sheep, before and after passage in vector midges (Diptera: *Ceratopogonidae*)" 4-7 April 2005, 156th SGM meeting - Edinburgh (UK)

"Live attenuated bluetongue vaccine viruses in a European breed of sheep, before and after passage in vector midges (Diptera: *Ceratopogonidae*)" 10-11th March 2005, Workshop EU funded research on Bluetongue and related diseases, Brussels (France)

"Replication of live attenuated BT vaccine virus in biting midges (Diptera: *Ceratopogonidae*) and the risks associated with their use in Europe". 11th of February 2005 Onderstepoort Veterinary Institute – Onderstepoort, Pretoria (South Africa). Presentation of the first year results on the Live attenuated vaccine project.

"Mosquito biology and control, with special reference to *Aedes albopictus:* the Italian experience Medical and Veterinary Entomology meeting (Royal Entomological Society Regional Meeting) in Durham (UK) on the 17th November 2004.

"Replication of live attenuated BTV 'vaccine' in biting midges (Diptera:*Ceratopogonidae*) and the possible risks associated with their use in Europe" The 3rd EMCA Workshop – Osijek (Croatia) 6th-9th October 2004

"Replication of live attenuated BTV vaccine in biting midges (Diptera: *Ceratopogonidae*) and the possible risks associated with their use in Europe". Royal Entomological Society National Meeting" 21-22nd July 2004 University of York (UK).

"Replication of live attenuated BTV2 vaccine in Dorset Poll sheep before and after passage through vector midges" Fourth coordination meeting –Lion (France) 10-11 June 2004.

"Development of a safe efficacious Bluetongue virus vaccination strategy for Europe". 1st Short Course for Young Parasitologists in Würtzburg, (Germany) 15-17th March 2004.

"3rd International meeting on European Funded Vaccine Project" New Forest (UK). 10th of October 2003.

Appendix 2

Andrew Shaw, **Eva Veronesi**, Guillemette Maurin, Najate Ftaich, Francois Guiguen, Frazer Rixon, Maxime Ratinier, Peter Mertens, Simon Carpenter, Massimo Palmarini, Christophe Terzian, and Frederick Arnaud.

"*Drosophila melanogaster* as a model organism for bluetongue virus replication and dissemination".
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Drosophila melanogaster as a Model Organism for Bluetongue Virus Replication and Tropism

Andrew E. Shaw, Eva Veronesi, Guillemette Maurin, Najate Ftaich, Francois Guiguen, Frazer Rixon, Maxime Ratinier, Peter Mertens, Simon Carpenter, Massimo Palmarini, Christophe Terzian and Frederick Arnaud

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Drosophila melanogaster as a Model Organism for Bluetongue Virus Replication and Tropism

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Bluetongue virus (BTV) is the etiological agent of bluetongue (BT), a hemorrhagic disease of ruminants that can cause high levels of morbidity and mortality. BTV is an arbovirus transmitted between its ruminant hosts by *Culicoides* biting midges (Diptera: Ceratopogonidae). Recently, Europe has experienced some of the largest BT outbreaks ever recorded, including areas with no known history of the disease, leading to unprecedented economic and animal welfare issues. The current lack of genomic resources and genetic tools for *Culicoides* restricts any detailed study of the mechanisms involved in the virus-insect interactions. In contrast, the genome of the fruit fly (*Drosophila melanogaster*) has been successfully sequenced, and it is used extensively as a model of molecular pathways due to the existence of powerful genetic technology. In this study, *D. melanogaster* is investigated as a model for the replication and tropism of BTV. Using reverse genetics, a modified BTV-1 that expresses the fluorescent mCherry protein fused to the viral nonstructural protein NS3 (BTV-1/NS3mCherry) was generated. We demonstrate that BTV-1/NS3mCherry is not only replication competent as it retains many characteristics of the wild-type virus but also replicates efficiently in *D. melanogaster* after removal of the bacterial endosymbiont *Wolbachia pipientis* by antibiotic treatment. Furthermore, confocal microscopy shows that the tissue tropism of BTV-1/NS3mCherry in *D. melanogaster* resembles that described previously for BTV in *Culicoides*. Overall, the data presented in this study demonstrate the feasibility of using *D. melanogaster* as a genetic model to investigate BTV-insect interactions that cannot be otherwise addressed in vector species.

Bluetongue virus (BTV) is an arbovirus belonging to the genus *Orbivirus* (family *Reoviridae*) that is biologically transmitted between its ruminants hosts by vector species of *Culicoides* biting midge (Diptera: Ceratopogonidae). In susceptible hosts, infection with BTV can lead to bluetongue (BT), a hemorrhagic disease of major importance for international trade and animal welfare (67). Historically, BTV has made only occasional incursions into Europe (46, 48, 73). Since 1998, however, BTV outbreaks have occurred virtually every year, resulting in severe economic losses across a wide geographic region (3, 45, 49, 78). Although severe clinical disease has been primarily restricted to improved wool and mutton breeds of sheep, the BTV-8 serotype, which entered Northern Europe in 2006 (15, 71), recorded relatively high case fatality rates in cattle (up to 1%) and a range of severe clinical signs (19, 50, 74, 78).

BTV is a complex nonenveloped virus with a 10-segmented double-stranded RNA (dsRNA) genome that encodes 7 structural proteins (VP1 to VP7) and 4 distinct nonstructural proteins (NS1, NS2, NS3/NS3A, and NS4) (5, 51, 60, 62). The virus particle is organized into three icosahedral protein capsids with an outer shell formed by VP2 and VP5, an inner capsid (or "outer core") composed of VP7, and an innermost layer (or "subcore") formed by VP3 (31, 32). The subcore surrounds the viral transcription complexes, composed of VP1 (polymerase), VP4 (capping enzyme), and VP6 (helicase) proteins, and the viral genomic segments (31, 61). The function of NS1 has yet to be fully defined, although it has been associated with cytopathogenesis (57) and the formation of characteristic tubules within the cytoplasm of infected cells (52). NS2 plays a key role in the formation of viral inclusion bodies (VIBs), where the assembly of new viral progeny takes place (11, 70). NS3/NS3A facilitates viral release, either by increasing plasma membrane permeability or by viral budding, according to the host cell considered (34, 39). NS4, the most recently described nonstructural protein of BTV, favors BTV replication in cells pretreated with interferon (5, 60).

Culicoides imicola is regarded as the major vector species in Africa (21) and Southern Europe (8, 48). It has been speculated that the progressive spread of this species in Europe is due to global warming and, in turn, is responsible for the increasing emergence of BTV in naïve European livestock (59). However, the recent BTV-8 outbreak in Northern Europe occurred beyond the northernmost limit of *C. imicola* (47), confirming earlier studies that had implicated Palearctic *Culicoides* species in the transmission of this virus (15). This hypothesis was later confirmed by the isolation of BTV from field-collected specimens that belong to the *Culicoides obsoletus* and *Culicoides pulicaris* groups, which are abundant in Central and Northern Europe (13, 20, 66), and the successful infections of both groups in the laboratory (14). In light of this evidence, the whole of Europe is currently regarded as "at risk" for the emergence of bluetongue and other arthropod-borne diseases (33, 44, 58).

Studies of *Culicoides*-orbivirus interactions have been hampered by the inability to successfully colonize the major BTV vec-

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tor species of Africa and Europe. Current understanding of the process of BTV infection and dissemination within the insect hosts are based almost entirely upon a single colonized species, *Culicoides sonorensis* (44). These studies have indicated that vector competence for BTV is determined in part by the presence of natural barriers to virus dissemination within adult *Culicoides* organisms. These barriers include (i) a mesenteron infection barrier (MIB) that controls the initial establishment of persistent gut infections upon BTV ingestion, (ii) a mesenteron escape barrier (MEB) that sequestrates BTV in gut cells, and (iii) a dissemination barrier (DB) that prevents infection of secondary organs, including salivary glands (24–26, 40, 46). Intrathoracic inoculation of adult *Culicoides* with BTV, however, leads to full dissemination of the virus and infection of secondary organs as a result of bypassing these barriers (9, 25, 40).

The lack of an accurately sequenced and annotated *Culicoides* genome and, consequently, the absence of genetic tools available for these organisms have restricted studies of *Culicoides*-orbivirus interactions. To date, the fruit fly (*Drosophila melanogaster*) genome is better characterized and understood than any other insect species, and it has been used in a vast array of studies of development and microbial pathogenesis, illustrating pathway conservation among vertebrates and invertebrates (17, 64, 72). Moreover, many of the classical signal transduction systems, including those involved in the immune response, were first identified in *D. melanogaster* using forward genetic screens (22, 43). More recently, it has become increasingly common to use *D. melanogaster* to study insect-pathogen interactions (17, 65). For instance, the bacterial endosymbiont *Wolbachia pipientis* (*w*Mel strain) of *D. melanogaster* increases resistance to infection by several RNA viruses, including many mosquito-transmitted arboviruses that are pathogenic in humans (6, 30, 36, 53, 69).

In this study, reverse genetics was used to generate a modified strain of BTV-1 expressing the mCherry fluorescent protein fused to NS3/NS3A (BTV-1/NS3mCherry). We demonstrate that BTV-1/NS3mCherry is able to replicate *in vitro* as well as *in vivo* in *C. sonorensis*, a competent vector for BTV. Furthermore, this virus was found to replicate efficiently *in vivo* in *D. melanogaster* after removal of *W. pipientis* infections by treatment with tetracycline. Finally, confocal analysis revealed that, similar to what has been observed in its insect vector (26), BTV replicates in the fat bodies, salivary glands, and proventriculi (foregut-midgut junction) of infected *D. melanogaster* flies.

MATERIALS AND METHODS

Cells. BSR cells (a clone of BHK-21) were kindly provided by Karl K. Conzelmann and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 25 μg/ml penicillin/streptomycin (P/S). Bovine fetal aorta endothelial (BFAE) cells were grown in Ham's F12 medium supplemented with 20% FBS and P/S. Both cell lines were incubated at 35 to 37°C in 5% CO_2. The KC cells used were originally derived from *C. sonorensis* embryos and were grown in Schneider's medium supplemented with 15% FBS and 25 μg/ml P/S at 25°C (77).

Plasmids and cloning. The 10 plasmids required to rescue the BTV-1 strain have been described previously (60). Each plasmid contains a single BTV genomic segment flanked by an N-terminal T7 promoter and a C-terminal restriction site to allow linearization and *in vitro* transcription of viral-like capped RNA. pUCBTV-1_Seg-10XhoI/EcoRVmod contains BTV-1 Seg-10 and was produced by site-directed mutagenesis inserting XhoI and EcoRV restriction sites into the region between the two predicted transmembrane domains of the NS3/NS3A protein (2). The mCherry coding sequence was amplified by PCR from pCMVRab5 WT-Cherry plasmid (55) using PfuUltra II Fusion HS DNA polymerase (Agilent). The PCR product was subsequently digested with XhoI and EcoRV and ligated into pUCBTV-1_Seg-10XhoI/EcoRVmod in order to obtain pBTV-1_Seg-10_mCherry. Detailed descriptions of primers and cloning procedures are available upon request.

BTV rescue. The wild-type (wt) BTV-1 and BTV-1/NS3mCherry were rescued by reverse genetics as already described (10, 60). Briefly, the rescue plasmids were digested with SapI or BsaI to generate exact 3′ termini of authentic BTV segments and were then used as the templates for *in vitro* transcription of BTV-like capped RNA. To rescue BTV, BSR cells were initially transfected with 1×10^{11} RNA copies of each segment encoding VP1, VP3, VP4, NS1, NS2, and VP6. After 18 h, the cells were further transfected with all 10 BTV segments, including the modified segment 10 encoding NS3mCherry. Transfection assays were performed using Lipofectamine 2000 (Invitrogen). Three hours after the second transfection, the medium was replaced with an agar overlay and cells were incubated at 35°C until plaques appeared. Individual plaques were then picked through the overlay, resuspended in 500 μl of DMEM, and used to infect BSR cells. Once the cytopathic effect (CPE) was advanced, the supernatant was separated from the cellular debris and stored as aliquots at 4°C. The cellular debris was resuspended in 1 ml TRIzol (Invitrogen), and the RNA was extracted according to the manufacturer's instructions. The dsRNA and single-stranded RNA (ssRNA) fractions were separated by precipitating the total RNA in the presence of 2 M lithium chloride.

The genome profiles of wt BTV-1 and BTV-1/NS3mCherry were analyzed by 1% agarose gel electrophoresis (AGE). Rescue assays were also performed and stained with crystal violet to assess the efficiency of virus rescue.

Confocal and electron microscopy. The day before infection, 1×10^5 BSR cells were plated in two-well glass chamber slides (LabTek) in 1 ml of growth medium. Subsequently, cells were infected with wt BTV-1 or BTV-1/NS3mCherry at a multiplicity of infection (MOI) of 0.001 by replacing the growth medium with 1 ml of virus diluted in DMEM (without serum) and incubated at 37°C for 24 h. The cells were then processed as already described (1, 54). Virus replication was detected using rabbit polyclonal anti-NS3 (for wt BTV-1) or anti-NS2 (for BTV-1/NS3mCherry) antibody. Goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes) was used as a secondary antibody. Slides were mounted with medium containing DAPI (4′,6-diamidino-2-phenylindole; Vectashield, Vector Laboratories) and analyzed with a Leica TCS SP2 confocal microscope.

For electron microscopy, BSR cells were plated in 3.5-cm-diameter dishes and infected at an MOI of 0.05 for 2 h at 37°C. At 24 h postinfection (p.i.), the cells were fixed for 1 h at 4°C with 2.5% gluteraldehyde and 1% osmium tetroxide and subsequently pelleted through 1% SeaPlaque agarose (Flowgen). Cells were embedded in Epon 812 resin, dehydrated in a graded alcohol series, and then "cut" and analyzed in a Joel 1200 EX II electron microscope.

Virus growth curves. BSR or BFAE cells (2×10^5) were plated in 12-well plates 1 day prior to infection. The cells were subsequently infected at an MOI of 0.05 for 2 h with the appropriate virus dilution in DMEM. KC cells (2×10^6) were plated in 12-well plates 1 day prior to infection. The cells were subsequently infected at an MOI of 0.005 for 2 h with the appropriate virus dilution in Schneider's medium. The inocula were then discarded, cells were washed with DMEM, and 1 ml of growth medium was added. Mammalian cells were then incubated at 37°C and KC cells at 28°C. At 0, 8, 24, 48, and 72 h p.i., 100 μl of supernatant was removed and replaced with 100 μl of fresh growth medium. The supernatant samples were clarified by centrifugation at $500 \times g$ for 5 min, and the cell-free fractions were titrated by limiting dilution assays in BSR cells as described previously (12, 60).

To determine the relative level of intracellular versus extracellular virus, 1×10^5 BSR or BFAE cells were first plated in 24-well plates. The next

day, the cells in multiple wells were infected at an MOI of 0.05 for 2 h. The inocula were discarded, the cells were washed, and 0.5 ml of complete medium was added. At 24 h p.i., before the appearance of CPE, the supernatant was harvested from two wells and stored at 4°C. The cell sheets were disrupted by freeze-thawing, and the cellular material was resuspended in 0.5 ml of DMEM. The supernatant and cellular fractions were clarified by centrifugation, and the supernatants were titrated by a limiting dilution assay. Each experiment was repeated four times. The ratios between intracellular and extracellular viral titers were calculated, and statistical analyses were performed with a Wilcoxon nonparametric test to compare two median percentages using R software (Comprehensive R Archive Network; http://www.R-project.org).

Western blotting. BSR cells were plated in 12-well plates and infected at an MOI of 0.01. After 24 h, the cells were harvested in 100 μl of sample buffer. Proteins were separated by SDS-PAGE, transferred to Amersham Hybond-P (polyvinylidene difluoride [PVDF]) membranes (GE Healthcare), and incubated for 1 h at room temperature with polyclonal rabbit antibodies against BTV NS1, BTV NS3, or a monoclonal mouse antibody against gamma tubulin (Sigma-Aldrich) as appropriate (60). Subsequently, the membranes were incubated for 1 h at room temperature in the presence of a horseradish peroxidase-conjugated secondary antibody (GE Healthcare). Finally, the membranes were washed and developed with ECL Plus (GE Healthcare), followed by exposure to X-ray film (GE Healthcare).

***Culicoides* infection assays.** *Culicoides sonorensis* adults were produced from the colony maintained at the Pirbright laboratory (7). Two- to 3-day-old females ($n = 60$) were intrathoracically injected with 0.2 μl of BTV-1/NS3mCherry (1.2×10^5 PFU/ml), wt BTV-1 (1×10^6PFU/ml) as a positive control, or Schneider's medium as a negative control and then incubated at 25°C in a netted waxed pillbox (Watkins and Doncaster, Stainton, United Kingdom) with a wet cotton pad on the top of it (5% sucrose solution) that was provided daily as a food source. At day 10 p.i., surviving females were collected for analysis. Each specimen was individually homogenized in a final volume of 1 ml of Schneider's medium supplemented with 1% P/S and 1% amphotericin B using a Qiagen TissueLyser as described previously (75). Homogenates from day 10 p.i. were individually titrated by limiting dilution assays in BSR cells (12, 60).

***D. melanogaster* and *Wolbachia*.** The wild-type Canton-S strain and the transgenic *D. melanogaster* strain (w; P{w+, GAL4-YP1.JMR}20) (here referred to as Yolk-Gal4) were maintained at 23°C on axenic medium. The P{GAL4-YP1.JMR} transgene can specifically direct expression of the yeast GAL4 transcription factor in the fat body cells of adult *D. melanogaster* females (28). The presence of *W. pipientis* in the *D. melanogaster* strains was assessed by PCR on DNA extracted from 10 individual *D. melanogaster* flies. Briefly, *D. melanogaster* flies were frozen at −20°C for at least 20 min and subsequently homogenized in 50 μl homogenization buffer (10 mM Tris-HCl [pH 8.2], 1 mM EDTA, 25 mM NaCl, and 200 μg/ml proteinase K). After 30 min of incubation at 37°C, proteinase K was inactivated by heating the samples for 10 min at 95°C. Cell debris was pelleted by centrifugation, and the resulting supernatant was stored at 4°C until assayed by PCR. 99F/994R and wsp81/wsp691 were used as primers to amplify 16S rRNA genes and *wsp*, respectively, from several strains of *W. pipientis* as described previously (80). The mitochondrial 12S rRNA gene was amplified with primer pair 12SAI/12SBI as described elsewhere (56). The presence of closely related *w*Mel strains (*w*Mel or *w*MelPop) was confirmed by sequencing PCR products. A Yolk-Gal4 *W. pipientis*-free line was generated by adding 0.25 mg/ml of tetracycline to the medium (38). After two generations of tetracycline treatment, *D. melanogaster* flies were grown for at least four generations on normal media in order for them to recover before being used for experiments. The Canton-S strain treated with tetracycline was kindly provided by the laboratory of Jean-Luc Imler. The absence of *W. pipientis* was assessed in individual *D. melanogaster* flies ($n = 10$) using PCR as described above.

***D. melanogaster* infection.** Two- to 3-day-old Yolk-Gal4 females, treated or not treated with tetracycline, were intrathoracically injected with 0.345 μl of BTV-1/NS3mCherry virus (4.5 $\log_{10}$ 50% tissue culture infective dose [$TCID_{50}$]/ml) or supernatant from uninfected BSR cells (referred to as mock infected). After injection, 5 *D. melanogaster* flies were either collected immediately (day 0) or incubated at 28°C for 10 days (day 10) before collecting and processing. Day 0 and day 10 *D. melanogaster* flies were individually homogenized in a final volume of 1 ml Schneider's medium containing 10% fetal bovine serum, 2.5 μg/ml amphotericin B, 100 units/ml nystatin, 50 μg/ml gentamicin, and 25 μg/ml P/S. The homogenate debris was pelleted by centrifugation, and the supernatant was retained for subsequent fluorescence-activated cell sorter (FACS) analysis. Each experiment was repeated three times, and 40 to 60 individuals were injected during each experiment.

In addition, 2- to 3-day-old *Wolbachia*-free Canton-S and Yolk-Gal4 females ($n = 60$) were intrathoracically injected with 0.345 μl of wt BTV-1 or BTV-1/NS3mCherry virus at the same viral titer (2×10^4 PFU/ml) or mock infected with supernatant from uninfected BSR cells. At day 0 and day 10 p.i., 10 females were collected and individually homogenized in a final volume of 1 ml DMEM containing 10% fetal bovine serum, 2.5 μg/ml amphotericin B, 100 units/ml nystatin, 50 μg/ml gentamicin, and 25 μg/ml P/S. Homogenates were then individually titrated by limiting dilution assays in BSR cells (12, 60).

FACS analysis. In order to detect BTV-1/NS3mCherry-positive cells, FACS analysis was performed on KC cells (1.5×10^5 cells/well) inoculated with 100 μl of individual *D. melanogaster* homogenate in 96-well plates. Experiments were repeated three times. The cells were incubated for 5 days at 28°C and then resuspended in 1% paraformaldehyde (PFA) prior to FACS analysis. Five days postinoculation of KC cells was optimal for discrimination between the homogenates of *D. melanogaster* flies collected at day 0 and day 10 postinfection.

Titrations by endpoint dilution in KC cells (1.5×10^5 cells/well) were also performed for the *D. melanogaster* homogenates producing the greatest number of mCherry-positive cells by FACS analysis. Briefly, a 10-fold dilution series of each homogenate was added to a 96-well plate (100 μl/well using four replicates per dilution). Plates were sealed and incubated at 28°C. At 7 days postinoculation, each well was analyzed by FACS and scored as positive if >0.5% of cells were mCherry positive (with 0.5% being the upper limit of the background obtained with mock-infected *D. melanogaster* homogenates). The virus titers were calculated as a 50% endpoint and expressed as $\log_{10}$ $TCID_{50}$/ml, calculated using the Spearman-Karber formula (23). FACS analyses were performed with a BD LSR II cytometer using BD FACSDiva 6.1.2 software. Forward scatter (FSC-A) and side scatter (SSC-A) data were used to characterize events corresponding to the viable KC cells. Statistical analyses were also performed with a Wilcoxon nonparametric test to compare two median percentages using R software.

Imaging of BTV-infected *D. melanogaster* flies. Mock- and BTV-injected tetracycline-treated *D. melanogaster* flies (Yolk-Gal4) were collected at day 10 p.i. and incubated in 2% PFA at 4°C for 24 h. *D. melanogaster* flies were rinsed in phosphate-buffered saline (PBS) and incubated in 20% sucrose solution overnight at 4°C before being frozen at −80°C in cryo-embedding media (OCT). Whole-fly cryosections (20-μm thick) were prepared and laid on SuperFrost Ultra Plus glass slides (Dutscher). Slides were subsequently mounted using a Vectashield mounting medium containing DAPI and analyzed by confocal microscopy using a Leica TCS SP5 microscope.

RESULTS

Rescue of BTV-1/NS3mCherry. By reverse genetics, we have designed and rescued a BTV-1-based virus expressing the mCherry fluorescent protein, located between the two predicted transmembrane domains of NS3/NS3A (Fig. 1A and B). This virus (identified as BTV-1/NS3mCherry) formed several plaques on BSR cell monolayers, although rescue efficiency was lower than that of wt BTV-1 (Fig. 1C). The genome segment migration patterns of wt BTV-1 and BTV-1/NS3mCherry were compared by 1% AGE. The

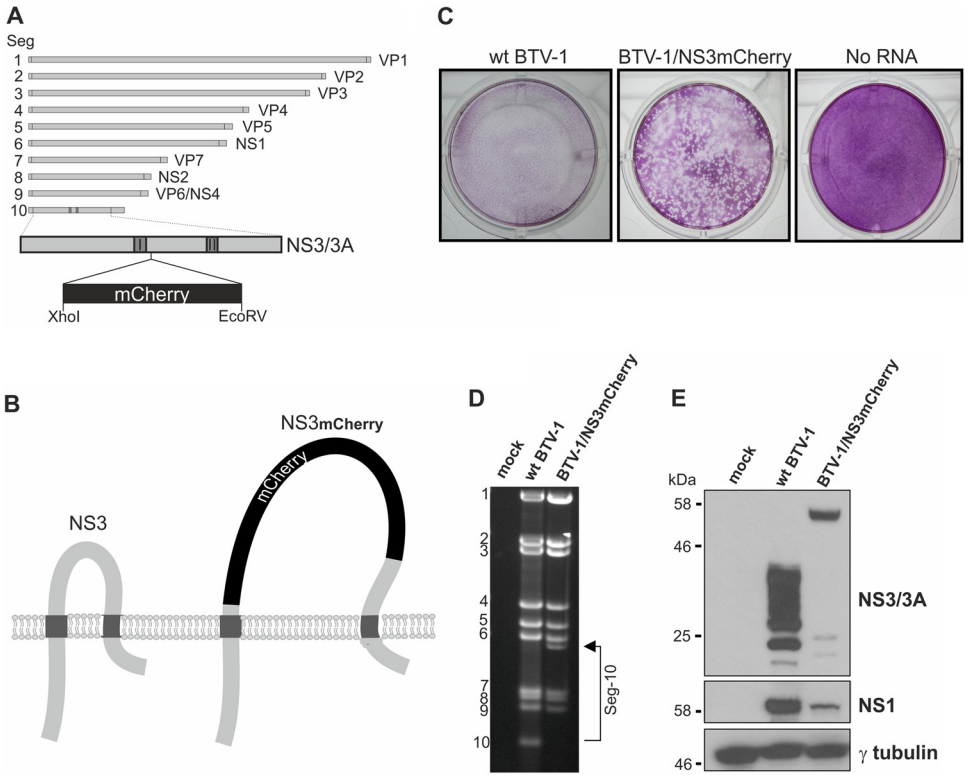

FIG 1 Rescue of a recombinant BTV-1 expressing the mCherry fluorescent protein. (A) XhoI and EcoRV sites were introduced into BTV-1 segment 10 (Seg-10) between the two transmembrane domains (I and II) of the NS3/3A protein. The restriction sites were then used to insert the mCherry fluorescent protein in frame with the NS3/NS3A coding sequence. (B) Based upon the predicted topology of the NS3 protein, mCherry is located in a loop on the noncytosolic side of the cell membrane (right), while leaving the cytosolic tails of the protein free to interact as per wild-type NS3 (left). (C) Crystal violet staining revealed that BTV-1/NS3mCherry was rescued efficiently from BSR cells. (D) wt BTV-1 and BTV-1/NS3mCherry display identical genomic profiles, except for Seg-10, which migrates at 822 and 1,530 nucleotides, respectively. (E) BSR cells were infected with wt BTV-1 or BTV-1/NS3mCherry, and lysates were analyzed by Western blotting with antibodies against viral NS1 and NS3/NS3A proteins. Note that, while the band corresponding to NS1 migrated at the same level for both wt BTV-1 and BTV-1/NS3mCherry, the band corresponding to NS3 migrated higher in BTV-1/NS3mCherry, due to the presence of the mCherry protein. Antibody against gamma tubulin was used as a loading control.

band corresponding to Seg-10 of BTV-1/NS3mCherry migrated more slowly than that of wt BTV-1, due to the presence of the mCherry coding sequence, confirming the incorporation of the modified Seg-10 into the BTV-1 genome (Fig. 1D). Protein bands were detected by Western blotting at 26 and 24 kDa (NS3 and NS3A, respectively), along with a smear at higher molecular weights typical of wt BTV-1 NS3/NS3A (Fig. 1E). In contrast, BTV-1/NS3mCherry displayed a band of approximately 53 kDa that corresponds to the NS3mCherry protein (Fig. 1E). NS1 was also detected in BSR cells infected with BTV-1/NS3mCherry, albeit at lower levels compared to wt BTV-1 NS1 (Fig. 1E).

Characterization of BTV-1/NS3mCherry. As mCherry is fused to NS3, a fluorescent signal is only observed when the recombinant virus enters the cell, replicates, and synthesizes its nonstructural proteins. By confocal microscopy, we observed mCherry expression in BSR cells infected with BTV-1/NS3mCherry. Moreover, the mCherry fluorescent signal was detected in the same cells as those expressing NS2 (Fig. 2A, panel ii), confirming that NS3mCherry is only expressed in cells with actively replicating BTVs. By electron microscopy, BSR cells infected with BTV-1/NS3mCherry displayed the hallmarks of BTV-infected cells, including the presence of NS1 tubules, VIBs containing progeny cores, and viral particles (Fig. 2B). Interestingly, viral particles budding at the plasma membrane were observed for wt BTV-1 but not BTV-1/NS3mCherry (Fig. 2B, panel iii).

Growth curves in BSR cells showed that BTV-1/NS3mCherry displays a slightly lower replication rate than wt BTV-1 (Fig. 3A). However, in BFAE and KC cells, wt BTV-1 replicated much more efficiently than BTV-1/NS3mCherry (Fig. 3B and C). In addition, the ratio between the intracellular and extracellular viral titers is significantly higher for BTV-1/NS3mCherry in BFAE cells (Wilcoxon sum of rank test, $P = 0.028$) than for BSR cells ($P > 0.05$), indicating a partial intracellular retention of the newly formed virion of BTV-1/NS3mCherry in this cell type compared to the wt BTV-1 (Fig. 3D and E). These data suggest that the function of NS3 on BTV egress (39) is partially compromised in the NS3mCherry fusion protein. Indeed, extensive passaging of BTV-1/NS3mCherry in BSR cells leads to the selection of deletion mutants that lack the intact mCherry reading frame (data not shown). However, BTV-1/NS3mCherry was able to replicate efficiently in injected *C. sonorensis* females (Fig. 3F).

wt BTV-1 and BTV-1/NS3mCherry replicate in *D. melanogaster*. Initial BTV-1/NS3mCherry infection assays in *D. melanogaster* were inconclusive, as the virus replicated only to low levels. We subsequently found that the Yolk-Gal4 *D. melanogaster* flies used for these experiments were positive for a strain of *W. pipientis*

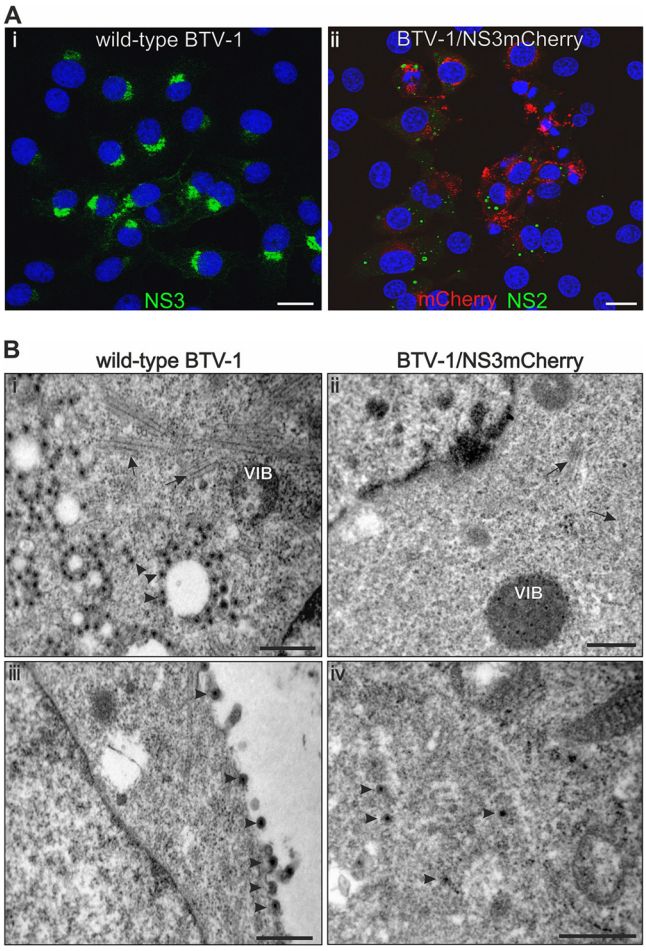

FIG 2 *In vitro* characterization of BTV-1/NS3mCherry. (A) Confocal microscopy of BSR cells infected with wild-type BTV-1 (i) or BTV-1/NS3mCherry (ii) at an MOI of 0.001 and fixed at 24 h p.i. Cells were immunolabeled using a polyclonal antiserum raised against NS3 (i) or NS2 (ii) and an Alexa Fluor 488 secondary antibody (shown in green) as described in Materials and Methods; BSR cells positive for BTV-1/NS3mCherry infection are shown in red (ii). NS2 immunolabeling indicates the presence of replicating viruses and was only observed in mCherry-expressing cells. Scale bars, 20 μm. (B) BSR cells infected with either wild-type BTV-1 (i and iii) or BTV-1/NS3mCherry (ii and iv) were fixed at 24 h p.i. and prepared for electron microscopy. Typical features associated with BTV-1 infection were observed, including viral inclusion bodies (VIB), NS1 tubules (arrows), and viral particles (arrowheads). Scale bars, 0.5 μm.

that is closely related to *w*Mel (Fig. 4A and data not shown) and known to increase the resistance to infection by several arboviruses (6, 30, 36, 53, 69). We therefore used tetracycline treatment to generate a *W. pipientis*-free line of *D. melanogaster* (Yolk-T) from the original, untreated Yolk-Gal4 stock (Yolk-NT). We were not able to detect *W. pipientis* by PCR in subsequent generations of *D. melanogaster* flies that had been treated with tetracycline, confirming the success of treatment (Fig. 4A). Yolk-Gal4 females, treated or not treated with tetracycline, were intrathoracically inoculated with BTV-1/NS3mCherry (or DMEM as a mock-infection control) and either collected immediately postinfection (D0) or incubated at 28°C for 10 days (D10) prior to collection (Fig. 4B). KC cells were infected with mock-infected or BTV-1/NS3mCherry-infected homogenates of day 0 and day 10 *D. melanogaster* flies and then incubated at 28°C for 5 days before analysis by FACS (Fig. 4B). KC cells inoculated with mock-infected homogenates displayed a background ranging from 0 to 0.4% of mCherry-positive cells at 5 days p.i. (regardless of whether or not the *D. melanogaster* flies had been treated with tetracycline) (Fig. 4C). Similarly, *D. melanogaster* injected with BTV-1/NS3mCherry and harvested immediately (day 0, input virus) yielded a low percentage (0.1 to 0.5%) of mCherry-positive cells, with a median value of 0.2% (Fig. 4C and D). KC cells infected with Yolk-NT homogenates from day 10 also displayed a low percentage of mCherry-positive cells, with a median value of 0.1%, indicating low levels of BTV replication (Fig. 4C and D). Together, these data reveal that Yolk-NT *D. melanogaster* failed to show any statistically significant BTV replication (as detected by mCherry signal) between day 0 and day 10 (Wilcoxon sum of rank test, $P = 0.156$) (Fig. 4D). In contrast, the Yolk-T strain consistently displayed high levels of BTV-1/NS3mCherry infection (36.2 to 62%), with a median value of 48.5% and a statistically significant increase in the levels of BTV replication between day 0 and day 10 ($P = 4 \times 10^{-6}$) (Fig. 4D). BTV titration assays by endpoint dilution in KC cells showed that the upper values in day 10 *D. melanogaster* homogenates is equivalent to 2.75 $\log_{10}$ $TCID_{50}$/ml for the Yolk-NT strain and 5.75 $\log_{10}$ $TCID_{50}$/ml for the Yolk-T strain (Fig. 4D).

Yolk-Gal4 is a transgenic strain that expresses a high quantity of the yeast transcription factor Gal4 in the fat body (28) and, therefore, may be less fit than other *D. melanogaster* strains. To this end, transgenic Yolk-T and wild-type Canton-S *Wolbachia*-free females (Fig. 4A) were intrathoracically inoculated with the same amount of wt BTV-1 or BTV-1/NS3mCherry at the same viral titer (2×10^4 PFU/ml) and either collected immediately postinfection or incubated at 28°C for 10 days prior to collection. Titration assays by endpoint dilution in BSR cells show a significant increase of the viral titers in day 10 Yolk-T and Canton-S *D. melanogaster* strains for both wt BTV-1 and BTV-1/NS3mCherry (Wilcoxon sum of rank tests, $P < 10^{-4}$ between day 0 and day 10 titers) (Fig. 4E and F). BTV-1/NS3mCherry replicated in injected *D. melanogaster* at a lower rate than wt BTV-1 ($P = 0.003$ in Yolk-T; $P = 0.005$ in Canton-S). Both wt BTV-1 and BTV-1/NS3mCherry displayed a lower replication rate in the Canton-S strain than in the Yolk-T *D. melanogaster* strain ($P = 0.009$ for wt BTV-1; $P = 0.036$ for BTV-1/NS3mCherry) (Fig. 4E and F).

BTV-1/NS3mCherry tropism. The fluorescence characteristics of BTV-1/NS3mCherry were used to identify tissues/organs in which BTV replication occurs in the *D. melanogaster* model. No mCherry signal was observed in mock-injected *D. melanogaster* (Fig. 5A to C), but an intense signal was detected in day 10 Yolk-T injected with BTV-1/NS3mCherry (Fig. 5D to O). The proventriculus (i.e., the junction between the foregut and midgut) was found to be heavily infected by BTV-1/NS3mCherry, with the fluorescent signal particularly restricted to the ectodermal cells of foregut origin (Fig. 5D to F). No signal was observed in the endodermal cells originating from the midgut (Fig. 5F). BTV-1/NS3mCherry replication was also observed in the salivary glands and fat body cells throughout *D. melanogaster*-infected individuals (Fig. 5G to O).

DISCUSSION

In this study, we demonstrate that *D. melanogaster* can be used as a highly malleable model for studies on BTV replication and tropism in insects. In addition, we developed a replication-compe-

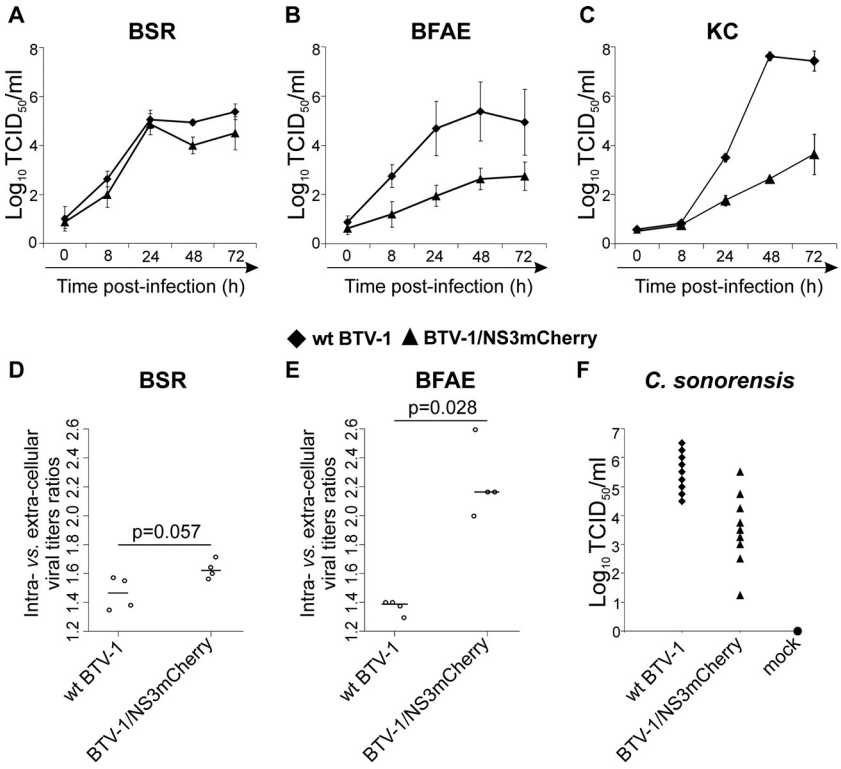

FIG 3 Replication rates of BTV-1/NS3mCherry. (A to C) *In vitro* virus growth curves were performed in BSR (A), BFAE (B), or KC (C) cells infected with wt BTV-1 or BTV-1/NS3mCherry. Multiple time points postinfection were generated by sampling and titrating cell supernatants. The BTV-1/NS3mCherry growth curve was more similar to that of wt BTV-1 in BSR cells than in BFAE or KC cells, where the increase in BTV-1/NS3mCherry titer was slower. (D to E) The graphs display the ratio between the intracellular versus extracellular virus titers in BSR (D) or BFAE (E) cells at 24 h p.i. BTV-1/NS3mCherry ratios are significantly higher than the wt BTV-1 in BFAE ($P < 0.05$) but not in BSR cells. (F) To determine whether BTV-1/NS3mCherry was able to replicate *in vivo*, *C. sonorensis* females were injected intrathoracically with BTV-1/NS3mCherry (or Schneider's medium as a negative control, referred to as "mock"). The wt BTV was used in this experiment as a positive control. After 10 days of incubation, *C. sonorensis* were individually homogenized and titrated by dilution assays on BSR cells. As indicated by the graph, BTV-1/NS3mCherry is able to replicate in *C. sonorensis*.

tent BTV expressing the mCherry fluorophore within the viral NS3 protein (BTV-1/NS3mCherry), which can be visualized by microscopy in a straightforward manner and will aid future research on vector competence in *Culicoides*. NS3 has been shown to be involved in a "late stage" of virus morphogenesis to orchestrate virus release and thus represents a good candidate for modification (16). Both the carboxy and amino termini of the NS3 protein have been shown to be important for virus-cell interactions (4); therefore, simply abutting a fluorescent protein to either terminus would reasonably be expected to disrupt one of these interactions. To overcome this issue, we inserted the mCherry protein in a site between the two predicted transmembrane domains of NS3/NS3A (2) and, by reverse genetics, we successfully rescued the modified BTV-1/NS3mCherry.

As expected, some differences between the NS3 expressed by the wild-type BTV-1 and the NS3mCherry expressed by BTV-1/NS3mCherry were observed. Both NS3 and NS3A proteins are encoded by the RNA Seg-10 and translated from two alternative ATG codons (63). By Western blotting, BTV-1/NS3mCherry displayed only one band of approximately 53 kDa, corresponding to NS3mCherry (Fig. 1E). NS3A expression is generally much lower than that of NS3 (79), most likely because both compete for the cell translation machinery. In addition, NS1 and NS3 protein levels appear lower in BTV-1/NS3mCherry than in wt BTV-1 (Fig. 1E), possibly due to the slightly different replication rates of these two viruses in BSR cells (Fig. 3A). It is therefore possible that the level of NS3AmCherry proteins is too low to be detected in our experiment. A few weak bands of a low molecular weight were also observed (Fig. 1E) and may represent degradation products. Moreover, the smearing of the wt BTV-1 NS3/NS3A, most likely associated with glycosylated forms of the protein (16, 79), was not observed (Fig. 1E), suggesting that glycosylation of NS3mCherry may be blocked. Indeed, the mCherry sequence is inserted close (6 amino acids) to the N-linked glycosylation site within NS3/NS3A. Based upon its role in viral egress, this alteration could affect NS3 function, which may in turn explain the difference observed in virus growth curves in BFAE and KC cells (Fig. 3B and C). Indeed, BFAE cells, like KC cells, failed to show CPE upon BTV infection, suggesting a mechanism of viral egress mainly by budding (60). Therefore, the differences in replication kinetics of wt BTV-1 and BTV-1/NS3mCherry *in vitro* (Fig. 3B and C) and *in vivo* (Fig. 4E and F) as well as the significant intracellular retention of newly formed BTV-1/NS3mCherry virions in BFAE cells (Fig. 3E) most likely represent a partial defect of NS3mCherry to mediate viral exit. Interestingly, the disruption or removal of glycans from proteins involved in cell exit has caused similar defects in virus release by other viruses, such as West Nile virus and Japanese encephalitis virus (35, 41). Nevertheless, BTV-1/NS3mCherry was shown to replicate *in vivo* in intrathoracically inoculated *C. sonorensis* and in

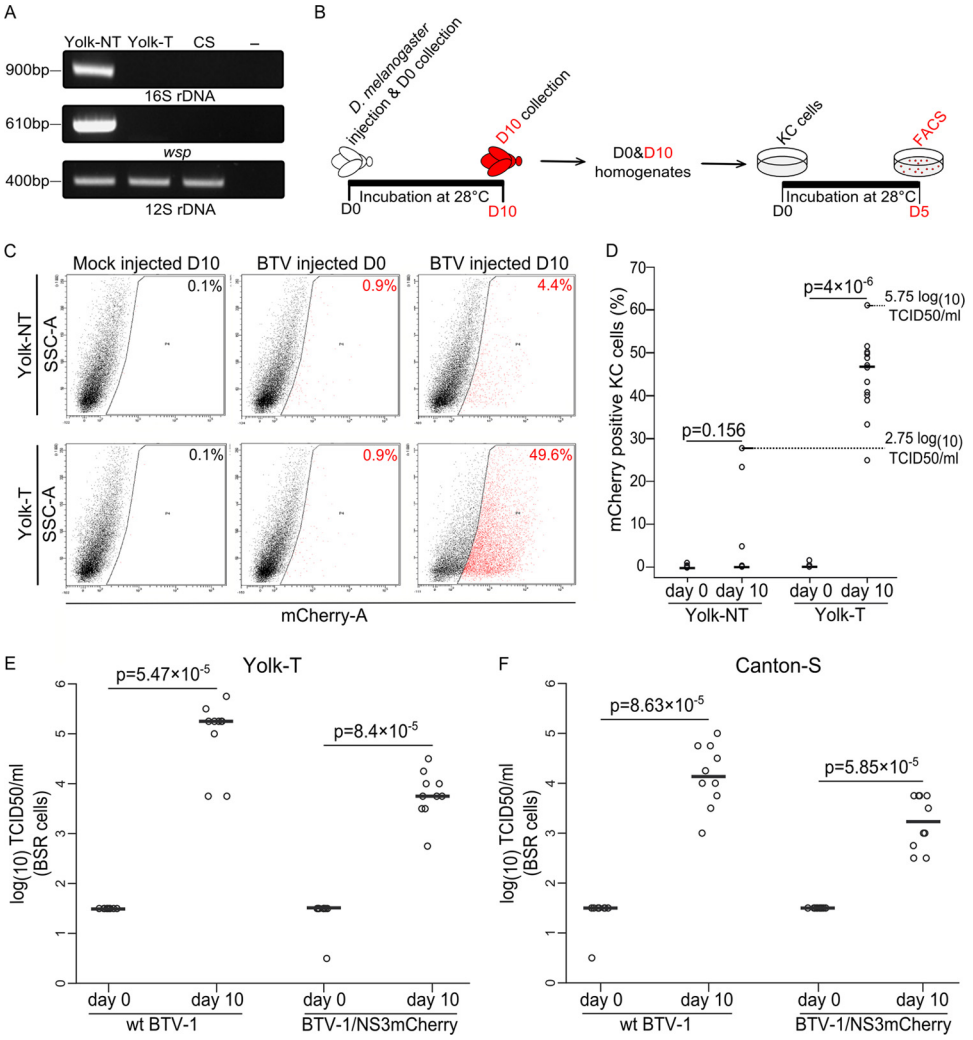

FIG 4 wt BTV-1 and BTV-1/NS3mCherry are able to replicate in *D. melanogaster*. (A) Specific PCR detection of *W. pipientis* before and after tetracycline treatment. Before tetracycline treatment, the Yolk-Gal4 strain (Yolk-NT) was found to be positive for *W. pipientis* using primers specific for 16S rRNA genes and *wsp*. In contrast, and as expected, the tetracycline-treated Yolk-Gal4 (Yolk-T) and Canton-S (CS) strains were found negative for *W. pipientis*. PCR amplification of mitochondrial 12S rRNA gene primers was used as a DNA extraction control. (B) Yolk-Gal4 *D. melanogaster* flies were injected with BTV-1/NS3mCherry and either harvested immediately (day 0, input virus) or incubated for 10 days prior to harvest (day 10). Each individual was homogenized and used to infect KC cells. Five days p.i., cells were analyzed by FACS. (C) Tetracycline-treated (Yolk-T) and untreated (Yolk-NT) *D. melanogaster* flies were injected with BTV-1/NS3mCherry (BTV) or virus-free medium (mock) and assayed as described above. *D. melanogaster* harvested immediately after injection (D0) showed very weak evidence of infection compared to those which had been mock injected (left versus middle panel). After 10 days of incubation (D10), the Yolk-T consistently showed high levels of infection, whereas the Yolk-NT showed only minimal evidence of viral replication (right panels). (D) The graph displays the percentage of mCherry-positive KC cells inoculated with *D. melanogaster* homogenates and harvested 5 days postinoculation. Horizontal bars represent the mean values of the data obtained from three independent experiments (5 individuals per day and per experiment). No significant difference was observed between day 0 and day 10 Yolk-NT homogenates ($P = 0.156$), whereas Yolk-T homogenates were highly permissive to BTV-1/NS3mCherry infection ($P = 4 \times 10^{-6}$). (E and F) Yolk-T (E) and Canton-S (F) *D. melanogaster* females were injected intrathoracically with wt BTV-1 or BTV-1/NS3mCherry at the same viral titer (2×10^4 PFU/ml) and either harvested immediately (day 0, input virus) or incubated for 10 days prior to collection (day 10). Each individual was homogenized and titrated by dilution assays on BSR cells. Horizontal bars represent the mean values of the data obtained (10 individuals per day and per experiment). All *P* values indicated between D0 and D10 titers are significant ($P < 10^{-4}$).

multiple cell lines, independent of any viral proteins provided in *trans*, while expressing a fluorescent form of NS3/NS3A.

The results reported in this study demonstrate that the wt BTV-1 and BTV-1/NS3mCherry replicate efficiently in both the transgenic Yolk-Gal4 and wild-type Canton-S *D. melanogaster* strains when introduced by intrathoracic inoculation. We noticed that BTV replication is significantly lower in Canton-S than in Yolk-Gal4. This difference may be explained by the fact that Yolk-Gal4 expresses a high quantity of the yeast transcription factor Gal4 in the fat body (28) and, therefore, may be less fit than wild-type Canton-S. Nonetheless, the Yolk-Gal4 strain may prove to be useful in future studies that require the specific knockdown of genes of interest in the fat bodies. During the course of the current study, evidence was also produced that *W. pipientis* can inhibit BTV replication in *D. melanogaster*. *Wolbachia* is a genus of bacteria that infects a high proportion of insect species, including *Drosophila* where it is widespread (18). It is recognized that certain species of *Wolbachia* are able to block infection by several patho-

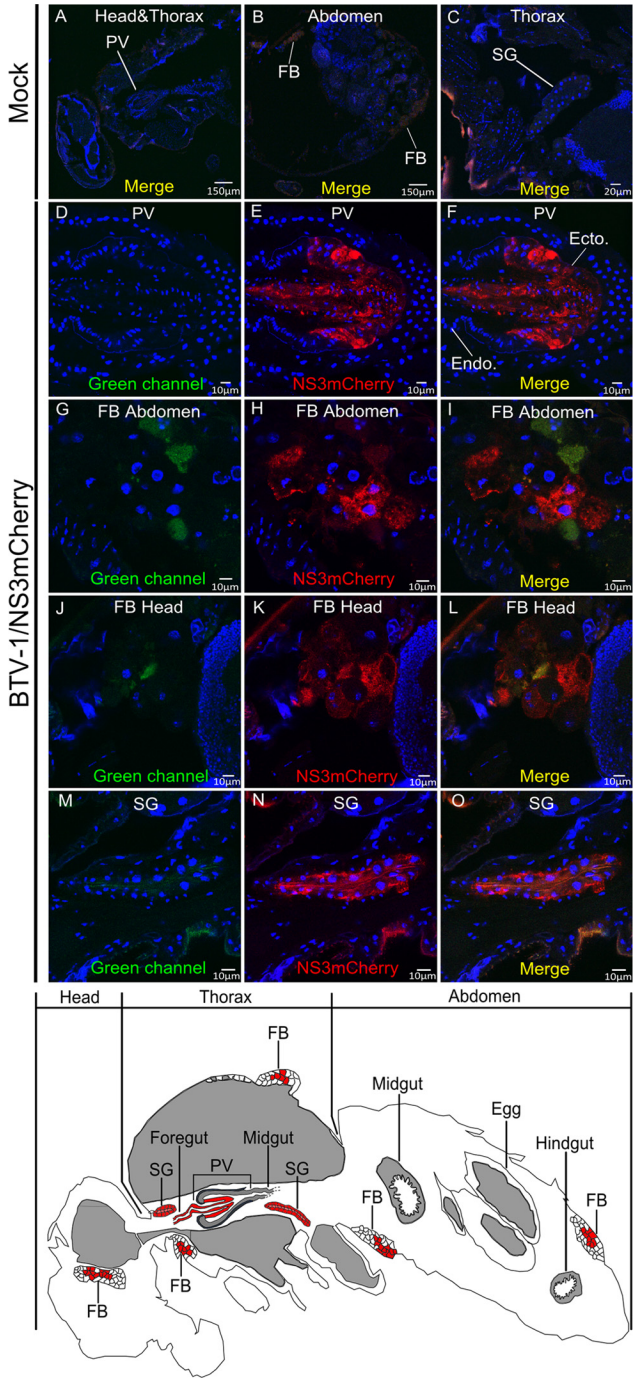

FIG 5 Confocal analysis of BTV-1/NS3mCherry tissue tropism. *D. melanogaster* (Yolk-T) flies were injected with BTV-1/NS3mCherry or virus-free medium (mock) and incubated for 10 days to allow viral replication. OCT-embedded flies were then sectioned and prepared for confocal analysis to assess BTV-1/NS3mCherry replication in tissues. (A to C) In all cases, the green channel is displayed to highlight tissue-derived background fluorescence. As expected, the mCherry signal was never detected in the head, thorax, or abdomen of mock-injected *D. melanogaster*. BTV-1/NS3mCherry efficiently replicated in the proventriculus (PV) (D to F), fat bodies (FB) of both the abdomen (G to I) and head (J to L), and the salivary glands (SG) (M to O) of infected *D. melanogaster* flies. (F) Note that in the PV, cells of ectodermal (Ecto) but not endodermal (Endo) origin were found positive for BTV-1/NS3mCherry replication. Scale bars, 10 μm. (Bottom) To illustrate the location of tissues/organs infected by BTV-1/NS3mCherry, a cartoon representing the sagittal section of *Drosophila* is shown.

gens, including members of the *Flaviviridae* (e.g., West Nile virus and dengue virus) (30, 76) and *Togaviridae* (e.g., chikungunya virus) (53) families. Our data show that BTV-1/NS3mCherry replicates efficiently only in tetracycline-treated *D. melanogaster* (which no longer contain detectable amounts of *W. pipientis*). Although this antibiotic treatment could potentially have other effects, our results suggest that the *W. pipientis*, in particular, a *w*Mel-related strain, is also able to inhibit BTV replication. Recent studies have demonstrated the use of the *w*Mel strain to control dengue fever in natural mosquito populations (37, 76). Future investigations will evaluate this approach to control animal pathogenic viruses transmitted by *Culicoides* vectors, such as BTV.

The natural route of BTV infection (via blood feeding) could not be applied in this study due to the different feeding behaviors of *Drosophila* (nonhematophagous) compared to *Culicoides* (hematophagous). The BTV-1/NS3mCherry virus was therefore injected directly into the insect hemocoel, bypassing any potential barriers to dissemination. In line with previously published data regarding BTV replication in *Culicoides* (26), we observed clear infection of fat bodies, salivary glands, and the foregut-midgut junction (proventriculus). BTV-1/NS3mCherry replication in the proventriculus was restricted to ectodermal cells (foregut origin), while the endodermal cells (midgut origin) were uninfected (27, 42). These data imply that BTV can enter from the basement membrane (e.g., membrane directly in contact with the hemocoel) of foregut cells but not midgut cells. Besides BTV, several other pathogenic arboviruses, including Venezuelan equine encephalitis virus and West Nile virus, have been shown to infect the foregut-midgut junction of their insect vectors (26, 29, 68). Therefore, infection of this organ may play a major role in the replication cycle of BTV in insects.

In summary, this study shows that reverse genetics can be used to generate BTVs expressing a viral protein tagged with a fluorescent molecule. We demonstrate that such a modified virus can replicate independently of helper cell lines and can be used for *in vivo* studies in an insect model. *D. melanogaster* offers a large array of well-developed molecular and genetic tools, which can be used to further investigate novel aspects of BTV-insect interactions that cannot be addressed in the natural vector species. In addition, BTV-1/NS3mCherry may also facilitate a better understanding of the role played by natural barriers in the modulation of species-specific susceptibility to BTV infection. Future experiments with orally infected midges using membrane-based blood-feeding techniques, which more closely resemble the natural route of infection, may reveal further details on BTV replication and dissemination within its insect vectors.

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Appendix 3

Veronesi E., P.P.C. Mertens, A. Shaw, P. Mellor and S. Carpenter. 2008.

"Quantifying bluetongue virus in *Culicoides* biting midges
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Quantifying Bluetongue Virus in Adult *Culicoides* Biting Midges (Diptera: Ceratopogonidae)

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ABSTRACT A TissueLyser system (QIAGEN) was used to rapidly and accurately estimate bluetongue virus "loads" in individual adult *Culicoides sonorensis* Wirth & Jones (Diptera: Ceratopogonidae). The optimized homogenization program that was developed, involved shaking insects for 1 min at 25 Hz with 2- or 3-mm stainless steel ball bearings. This program was used to measure the quantities of bluetongue virus present in insects that had either been inoculated or had ingested a viremic bloodmeal through an artificial membrane. The virus titers obtained using either infection technique and the optimized program did not differ significantly from those obtained using a polypropylene motor-driven pestle, a method that is currently in common use for studies of vector competence). The advantages of the new method, as a rapid means of detecting fully disseminated infections in individual field-caught flies, are discussed. Its use is compared with the processing of pools of *Culicoides* by conventional methods, where the extent of dissemination of the virus is unknown and could wrongly implicate species that are of low importance in transmission.

KEY WORDS *Culicoides*, Bluetongue virus, vector competence, oral susceptibility, TissueLyser

Bluetongue virus (family *Reoviridae*, genus *Orbivirus*, BTV) is transmitted between ruminants primarily via the bite of certain species of *Culicoides* (Diptera: Ceratopogonidae) biting midges. The detection of BTV in field-caught adults can be used to implicate certain species as vectors, information that is vital to our understanding of bluetongue disease epidemiology. Studies of vector competence in the northern Palaearctic and Mediterranean regions have relied on virus isolation, or reverse transcription-polymerase chain reaction (RT-PCR) detection of viral RNA. These methods have been used to analyze pools of parous, nonengorged female *Culicoides*, caught "at light" on farms where recent transmission has occurred (e.g., Mellor and Pitzolis 1979, Caracappa et al. 2003, De Liberato et al. 2005, Ferrari et al. 2005, Savini et al. 2005, Mehlhorn et al. 2007). However, these techniques take no account of the proportion of *Culicoides* that have fed on viremic animals, but, due to the presence of internal barriers to virus dissemination within the insect, subsequently develop only a nontransmissible although persistent infection (Jennings and Mellor 1987, Fu et al. 1999). The proportion of adult *Culicoides* from European species that becomes infected in this way is currently unknown, although a laboratory-based study suggests that it may be high (Carpenter et al. 2006).

Recently, a high-throughput TissueLyser/RT-PCR system was used to amplify RNA from individuals and pools of *Culicoides sonorensis* Wirth & Jones that were either intrathoracic (i.t.) inoculated or orally infected with BTV (Kato and Mayer 2007). Similar methods are in common use as part of other arboviral surveillance programs (Shi et al. 2001, Nasci et al. 2002). The length of time taken by "noncompetent" adult *Culicoides* to clear virus from their gut after they have fed on a viremic host (to give a negative RT-PCR result) is currently unknown. This issue is further complicated by the very high sensitivity of RT-PCR techniques and their ability to detect both infectious virus, and inactivated particles that could survive for sometime within the insect gut. However, it has been reported that individual adult *C. sonorensis* containing >3 $\log_{10}$ 50% tissue culture infected doses ($TCID_{50}$) of BTV per insect, will have a fully disseminated infection (Fu et al. 1999). A method that can be used to rapidly and accurately estimate viral load in terms of the amount of infectious virus per insect would, therefore, be very valuable for vector competence studies. In this study, we have optimized a TissueLyser method for the homogenization, isolation, and quantification of BTV from individual adult female *Culicoides*. The efficiency of the TissueLyser was also compared with that using the standard grinding method (polypropylene motor-driven pestles).

Materials and Methods

Virus and *Culicoides*. All infection experiments were carried out using a Kosovo strain of BTV-9 (KOS2001/03) that had been passaged twice through eggs and four times through BHK-21 cell culture (for

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further details on the virus, see http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-9). The infectivity of the initial virus preparation (tissue culture supernatant) was measured in BHK-21 cells, as 6.75 $\log_{10}$ $TCID_{50}$ per ml. Groups of 2–3-d-old colonized *C. sonorensis*, a North American BTV vector, from the PIRB-s-3 strain (Wellby et al. 1996) were infected by using semiautomatic i.t. inoculation (Boorman 1975), or by membrane-feeding on a 1:1 volume mixture with sheep blood (Mellor 1971). Inoculated midges were processed immediately after inoculation, whereas membrane-fed adults were immobilized using CO_2 and the fully engorged female collected, placed in a pillbox (Watkins & Doncaster, Cranbrook, Kent, United Kingdom), and incubated at 25 ± 1°C at 80% RH. A cotton pad moistened with a 5% sugar solution was provided daily. After 10-d incubation, surviving females were selected for use.

Homogenization of Samples. The TissueLyser (QIAGEN, Crawley, United Kingdom) is capable of simultaneously homogenizing 48 samples by using high-frequency shaking of each sample with a ball bearing (Dejay Distribution Ltd., Crowborough, United Kingdom). Initially, seven programs of varying duration and frequency of homogenization (based around the manufacturer's recommendations), were tested with 10 i.t. inoculated *C. sonorensis* each (Table 1). Before homogenization, the adult insects were transferred into a 1.5-ml Eppendorf tubes containing 100 μl of Glasgow minimal essential medium (MEM; with 0.6% field antibiotics containing 2.0 μg/ ml Fungizone, 1,000 IU/ml penicillin, 50 mg/ ml neomycin, and 1,000 IU/ml polymyxin). After grinding with 3-mm-diameter stainless steel ball bearings, each ball was removed from the tubes using a magnet, 900 μl of Glasgow MEM was added, and the tubes were centrifuged at 13,000 × *g* for 5 min. Virus titrations of the supernatant were carried out on BHK-21 cell monolayers in 96-well microtiter plates, looking for cytopathic effect at three and 5 d postinoculation (as described in Carpenter et al. 2006). The results of titer calculations were expressed as $\log_{10}$ $TCID_{50}$ per ml (Finney 1964). The presence of BTV was confirmed in all positive samples using a real-time RT-PCR assay (Shaw et al. 2007) and enzyme-linked immunosorbent assay (ELISA) (Thevasagayam et al. 1996).

An additional 10 i.t.-inoculated *C. sonorensis* also were ground using the earlier conventional method. In each case, power-driven polypropylene pestles were used in Eppendorf tubes for 30 s, and virus quantity was then determined by titration in an identical manner. After Levene's test for inequality of variance among the treatments used, the Kruskal–Wallis analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) was used to differentiate median titers among the eight treatments (all statistical testing performed using MINITAB version 14, Minitab, Inc., State College, PA).

After this initial experiment, the effect of ball bearing size and construction material were investigated using stainless steel balls measuring 1, 2, 3, 4, and 5 mm in diameter and stainless steel versus polyethylene balls at 3-mm diameter only (Dejay Distribution Ltd.). For the former experiment, 10 i.t.-inoculated individuals were ground with each ball bearing size for 1 min at 25-Hz frequency. In the later experiment 10 i.t.-inoculated individuals were ground using 3-mm stainless steel or polyethylene balls at a variety of frequencies (Table 1). Virus presence and quantification was carried out as described previously, and statistical analyses were carried out in the same way for the ball diameter treatments using Mann–Whitney *U* test for stainless steel versus polyethylene treatments.

Table 1. Mean virus titer of intrathoracically inoculated *C. sonorensis* ground with stainless steel and polyethylene balls for different periods of time (minutes) and at different frequencies (Hz)

Treatment time/ frequency (Hz)	Mean virus titer ($\log_{10}$ $TCID_{50}$) ± 95% CI (n = 10 for each treatment): stainless steel balls	Mean virus titer ($\log_{10}$ $TCID_{50}$) ± 95% CI (n = 10 for each treatment): polyethylene balls
1 min/25	2.58 ± 0.26^a	1.90 ± 0.33^c
30 s/20: 30 s/30	2.48 ± 0.20^a	1.88 ± 0.21^c
2 min/25	2.15 ± 0.33^a	1.35 ± 0.35^c
1 min/25: 1 min/30	2.03 ± 0.29^a	1.32 ± 0.25^c
2 min/25: 1 min/30	1.08 ± 0.26^b	
2 min/25: 2 min/30	0.97 ± 0.28^b	
4 min/30	0.53 ± 0.41^b	
Power pestle	2.45 ± 0.40^a	

[a, b] Pairwise significant difference in groups recorded using Tukey HSD at $P < 0.05$.

[a, c] Pairwise significant difference in values recorded for each processing treatment using Mann–Whitney U at $P < 0.05$.

Validation of Optimized Technique and Sensitivity with Pooled Samples. To validate the method, 40 i.t.-inoculated midges were individually ground with 2-mm stainless steel balls for 1 min at 25 Hz and compared with 40 insects ground via the power-driven pestle method. Additionally, two groups of 80 *C. sonorensis* that had been orally fed virus/blood suspension and incubated for 10 d (as described previously) were ground individually with the TissueLyser, or by using the motorized pestle method, and their virus titer measured in an identical manner. Differences in titer were again analyzed using Mann–Whitney *U* test.

Virus detection also was carried out using pools of midges with different [virus positive:virus negative] ratios (1:1, 1:5, 1:10, 1:25, 1:50, and 1:100). Intrathoracic-inoculated midges were incubated for 7 d at 25°C and 80% RH to allow full dissemination of the virus infection. The pools of insects were ground in a 1.5-ml Eppendorf tube, as described previously using the 2-mm stainless steel balls for 1 min at 25 Hz and then 2 min at 30 Hz. Supernatants were titrated on BHK-21 cells (four replicates per pool). In all cases, ELISA and RT-PCR assays were used to confirm the presence of the virus.

Results

A significant difference in the amount of virus obtained was observed between different grinding programs (H = 57.73, df = 7, $P < 0.001$), although no significant difference was noted in variance (W =

Table 2. Mean virus titer of intrathoracically inoculated *Culicoides sonorensis* ground with stainless steel balls of different diameters (mm) for 1 min at 25-Hz frequency

Ball bearing diam. (mm)	Mean virus titer ($\log_{10}$ $TCID_{50}$) ± 95% CI (n = 10 for each treatment)
1	0.70 ± 0.45[a]
2	1.85 ± 0.21[b]
3	1.54 ± 0.35[b]
4	1.42 ± 0.35[b]
5	0.74 ± 0.36[a]
Power pestle	1.55 ± 0.34[b]

[a, b] Pairwise significant difference in groups recorded using Tukey HSD at $P < 0.05$.

1.18, $P = 0.325$). Optimal grinding was achieved using relatively short runs in the TissueLyser (Table 1). Four of the programs used showed no significant difference when compared with the standard power pestle method, and treatment of 1 min at 25 Hz gave the highest mean virus titer.

The use of different diameter stainless steel ballbearings resulted in the recovery of significantly different quantities of virus ($H = 22.79$, df = 5, $P < 0.001$). Of those ball-bearing sizes tested, 1- and 5-mm diameter were significantly less effective than 2, 3, and 4 mm, which gave titers that did not differ significantly from those obtained using the pestle method (Table 2). Stainless steel balls also performed significantly better than the polyethylene balls ($P < 0.01$ in all cases; Table 1).

Homogenizing a further 40 i.t.-inoculated *C. sonorensis* by using the optimized combination of 2-mm ball bearings with a 1-min, 25-Hz grinding program gave titers that were not significantly different from those obtained for 40 individuals ground by using the powered pestle method (TissueLyser, 1.48 ± 0.18 [mean ± 95% CI]; power pestle, 1.60 ± 0.19; $W = 1526$, $P = 0.368$). Thirty-five of the 80 *C. sonorensis* that were infected by membrane feeding contained virus at 10 d postinfection, of which 25 contained >3 $\log_{10}$ $TCID_{50}$ per insect. Thirty-eight of the 80 *C. sonorensis* homogenized using motorized pestles contained virus, of which 23 contained >3 $\log_{10}$ $TCID_{50}$ per insect. The maximum titer achieved using both methods was 5 $\log_{10}$ $TCID_{50}$ per insect, and no significant difference was found between the two methods in the mean titer of those midges containing >3 $\log_{10}$ $TCID_{50}$ per insect ($W = 620.5$, df = 44, $P = 0.876$). Virus was detected in all the pools of midges with titers ranging from 4 $\log_{10}$ $TCID_{50}$ per pool to 6.75 $\log_{10}$ $TCID_{50}$ per pool. All the above-mentioned results were confirmed as positive by real-time RT-PCR and indirect sandwich ELISA.

Discussion

The identification of vector(s) involved in arboviral outbreaks is essential for the design and application of appropriate control programs. Conventional methods of identifying vector species, involving detection of virus and virus replication in pools of insects, have many drawbacks, and the labor involved in the large-scale screening of individuals for transmissible infections has restricted their use. We have demonstrated the recovery of infectious BTV from both i.t.-inoculated and membrane-fed *C. sonorensis* by using 2- or 3-mm steel ball bearings and an optimized TissueLyser grinding program of 1 min at 25 Hz. The titers of the recovered virus were statistically indistinguishable from those obtained using the current standard technique of grinding with polypropylene pestles (as described by Venter et al. 2005 and Carpenter et al. 2006). Although the preparation time of individual samples for grinding is marginally increased by adding stainless steel balls to Eppendorf tubes containing the samples, the homogenization step itself is ≈24 times faster (the TissueLyser can process 48 samples simultaneously).

The method not only can identify potential vectors more rapid and accurately but also uses stainless steel balls that are inexpensive and disposable, removing any possibility of contamination via the reusable pestles. By using a standardized homogenization program, variations caused by different operators by using different conditions to grind individual insects also are removed, potentially increasing the comparability of data between laboratories.

Due to difficulties in successfully feeding northern Palaearctic species of *Culicoides*, this study used adults of *C. sonorensis* as a model species of vector (Venter et al. 2005). However, it seems likely that the technique will be applicable to other *Culicoides* species, and potentially for other orbiviruses, including African horse sickness virus and epizootic hemorrhagic disease virus. The use of real-time RT-PCR is currently limited to a simple confirmation that test or diagnostic samples contain BTV. However, once a better understanding of the relationships between threshold cycle values, virus titer and transmissibility of infection are established, it may be possible to replace virus isolation/titration with real-time RT-PCR assays (which are more rapid and have the potential for a much higher throughput). This would increase the speed of processing substantially. Pairing of the technique with PCR assays for species identification (e.g., for the northwestern Palaearctic species; Nolan et al. 2007, Mathieu et al. 2007) also should prove to be a straightforward task, given the sensitivity already achieved with these methods and the fact that identification of DNA from different insect species would not require quantification.

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Appendix 4

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Temperature Dependence of the Extrinsic Incubation Period of Orbiviruses in *Culicoides* Biting Midges

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Abstract

Background: The rate at which viruses replicate and disseminate in competent arthropod vectors is limited by the temperature of their environment, and this can be an important determinant of geographical and seasonal limits to their transmission by arthropods in temperate regions.

Methodology/Principal Findings: Here, we present a novel statistical methodology for estimating the relationship between temperature and the extrinsic incubation period (EIP) and apply it to both published and novel data on virus replication for three internationally important orbiviruses (African horse sickness virus (AHSV), bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV)) in their *Culicoides* vectors. Our analyses show that there can be differences in vector competence for different orbiviruses in the same vector species and for the same orbivirus in different vector species. Both the rate of virus replication (approximately 0.017-0.021 per degree-day) and the minimum temperature required for replication (11-13°C), however, were generally consistent for different orbiviruses and across different *Culicoides* vector species. The estimates obtained in the present study suggest that previous publications have underestimated the replication rate and threshold temperature because the statistical methods they used included an implicit assumption that all negative vectors were infected.

Conclusions/Significance: Robust estimates of the temperature dependence of arbovirus replication are essential for building accurate models of transmission and for informing policy decisions about seasonal relaxations to movement restrictions. The methodology developed in this study provides the required robustness and is superior to methods used previously. Importantly, the methods are generic and can readily be applied to other arbovirus-vector systems, as long as the assumptions described in the text are valid.

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Introduction

Arboviruses that utilise propagative biological transmission require a period of replication and dissemination within the arthropod vector (the *extrinsic incubation period*, EIP) [1]. As arthropods are poikilothermic, the temperature of their environment is a key factor affecting the rate at which an arbovirus is able to replicate to transmissible levels in a vector following ingestion. In the case of arboviruses which have recently emerged into new regions, such as bluetongue virus (BTV), a better understanding of the relationship between environmental temperature and virus replication is essential when trying to predict geographical and seasonal limits of transmission [2,3,4]. Furthermore, this information could inform policies currently used to limit the economic impact of an outbreak, such as the timing of animal movement restrictions, which are currently based on entomological activity [5].

Orbiviruses are vector-borne pathogens transmitted between vertebrate hosts by haematophagous arthropods. Three orbivirus species, all of which are transmitted by *Culicoides* biting midges, are of particular importance: BTV, African horse sickness (AHSV) and epizootic haemorrhagic diseases virus (EHDV). BTV causes a non-contagious, infectious disease called bluetongue (BT) in ruminants, particularly improved breeds of sheep. This disease has come to particular attention over the past decade following an unprecedented series of economically damaging outbreaks in Europe [6,7,8]. African horse sickness is a disease of equids caused by AHSV, which rarely causes clinical disease in donkeys or zebra, but can cause mortality of up to 90% in horses [9,10]. Finally, epizootic haemorrhagic disease (caused by EHDV) often results in death in white-tailed deer (*Odocoileus virginianus*) and, less frequently, a bluetongue-like illness in cattle [11].

Several previous studies have examined the replication of orbiviruses in *Culicoides* incubated at controlled temperatures under laboratory conditions [12,13,14,15,16]. However, these studies relied on small numbers of insects, a problem compounded by the fact that a high proportion of individuals in any *Culicoides*

population may be incapable of developing a fully disseminated infection [17,18]. Furthermore, the statistical methods used to analyse the data were not ideal and did not fully reflect the experimental design. Finally, virus strains used in some previous studies were subjected to a relatively high degree of tissue passage, which may alter their ability to replicate in the insect vector [19,20].

In this paper we develop novel statistical methods for analysing the temperature dependence of the EIP of arboviruses, which correctly reflect the experimental design and allow rigorous comparison of differences in replication rate amongst orbiviruses and vector species. These methods are applied to new experimental data in which a substantial number of *Culicoides sonorensis* (a confirmed vector species) were experimentally infected with a strain of BTV subjected to relatively few tissue passages. The methods are also applied to previously published data on orbivirus replication [14,15] and the resulting parameter estimates are compared with those derived previously [15,16]. We discuss limitations to previous methodologies used to characterise the EIP in *Culicoides*-orbivirus systems (in addition to those pointed out by [16]) and compare them with methods used for mosquito vectors. Finally, we discuss the application of modelling in the implementation of control measures against arboviral spread with reference to the recent BTV outbreak in northern Europe.

Materials and Methods

Laboratory determination of the EIP of BTV-9 (Kosovo) in *Culicoides sonorensis*

Groups of 2-3 day old *C. sonorensis* from the PIRB-s-3 strain [21] of the Pirbright colony [22] were fed on a blood-virus suspension containing 1 mL of heparinized sheep blood (TCS Biosciences Ltd, UK), and 1 mL of BTV supernatant. A BTV serotype 9 strain isolated in Kosovo (sample KOS2001/02 in the EU community reference laboratory collection; http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-9.htm) was used. This strain was selected for study as it represented the most northerly outbreak in Europe prior to the current BTV-8 epizootic and had been previously demonstrated to be capable of infecting UK populations of *Culicoides* [18]. The isolate had been subjected to a single passage through an 11 day old embryonated chicken egg and three passages through BHK-21 cells, and was used at a titre of $10^{6.5}$ tissue culture infectious dose 50 ($TCID_{50}$)/mL when combined with blood in equal volume. Use of the chick embryo was conducted under Home Office project licence PPL 70/5793.

Adult midges were blood-fed using an artificial feeding apparatus [23] , with a stretched membrane of Parafilm® (Cole-Parmer, UK) used in place of the chick skin in the original method. *Culicoides* were allowed to feed for 30 min and then anaesthetised briefly with CO_2 to remove and discard non-feeding females and males. Blood-fed females were then placed in netted, waxed pill-boxes (Watkins & Doncaster, UK) and stored in incubators at temperatures of 15, 20, 25 and 30°C. The incubating *C. sonorensis* females were provided with 5% sucrose via a cotton wool pad applied to the netting and changed daily. At 15, 20, 25 and 30°C a sample of 25 females was removed immediately after the blood meal and then every day until too few were left for further sampling (10–23 days depending on temperature). In a subsequent experiment carried in a methodologically identical fashion but using 12°C incubation, a sample of 25 females was removed immediately after the blood meal and then every five days for 40 days.

Following incubation, individual midges were placed in autoclaved 1.5 mL Eppendorf tubes containing 100 µL of chilled Glasgow minimal essential medium (MEM) with 0.6% antibiotics (2.0 µg/mL Fungizone, 1000 IU/mL penicillin, 50 mg/mL neomycin, and 1000 IU/mL polymyxin). They were then homogenized using sterilized, motor-driven, polypropylene pestles. Nine hundred µL of MEM was then added to each sample, and the tubes were centrifuged at 12,000 *g* for 5 min. One hundred µL of the resulting supernatant was then used to prepare a 1:10 diluted sample using MEM in an additional Eppendorf tube for each sample. Virus titrations were carried out in tissue culture grade, 96-well microtitre plates containing a monolayer of BHK-21 cells and 100 µL of MEM supplemented with 2% tryptose phosphate broth (Invitrogen, United Kingdom) and antibiotics. Then, 100 µL of each sample and of 1:10, 1:100 and 1:1000 dilutions of each sample were inoculated onto plates in four replicates, together with a positive control of the original virus used (at 10^{-4} to 10^{-7} dilution), and a negative control of diluent. Plates were sealed and incubated in an incubator at 37°C with microscopic observation for cytopathic effects at 3 and 5 days. Where bacterial contamination occurred, samples were passed through a 0.2 µL disposable filter (Sartorius UK) using a 5 mL syringe. Titres of positive samples were calculated using the method of Spearman and Kärber [24]. *Culicoides sonorensis* females containing $\geq$2.5 $\log_{10}$ $TCID_{50}$ of BTV were considered to have replicated the virus to transmissible levels [25,26].

Previously published data on the EIP of orbiviruses

Further data were extracted from two papers: one on the replication of four orbiviruses (AHSV-4, BTV-10, BTV-16 and EHDV-1) in colony *C. sonorensis* [15]; and one on the replication of BTV-1 in field-collected *C. bolitinos* and *C. imicola* in southern Africa [14]. The latter were re-analysed from original datasets and times to transmissible infections interpreted from the proportion of *Culicoides* possessing $\geq$2.5 $\log_{10}$ $TCID_{50}$ of BTV/individual and the number and titre of infected midges during the previous time period.

Data from two other papers were not analysed. The first presented an investigation of the temperature dependence of the EIP of BTV-11 in colony *C. sonorensis* AK strain [12], but completion of the EIP was judged by ELISA absorbance values (correlated originally to plaque assay quantification) [27] rather than virus titres, making results difficult to compare. The second collated data on the EIP derived from a range of sources (see references in [13]) , but most investigated virus replication at a single temperature using a variety of different viruses and methods of virus detection. All the data used in the present analysis are summarised in Table 1.

Modelling the relationship between EIP and temperature

The EIP was assumed to follow a gamma distribution with probability density function given by,

$$f(u;v,k)=\frac{kv\mathrm{e}^{-kvu}(kvu)^{k-1}}{\Gamma(k)},\quad u\geq 0, \tag{1}$$

where ν is the reciprocal of the mean EIP and k is the scale parameter (so that $1/\nu$ and $1/k\nu^2$ are the mean and variance for the distribution, respectively). The temperature dependence of the EIP is reflected in the reciprocal of the mean (i.e. ν) which is given by,

$$v=v(T)=\begin{cases}0 & T\leq T_{\min},\\ \alpha(T-T_{\min}) & T>T_{\min},\end{cases} \tag{2}$$

Table 1. Summary of datasets on the EIP of orbiviruses and vector species used in this study.

orbivirus*	vector species	source	temperatures (°C)	N†	reference
AHSV-4	*C. sonorensis*	colony	15, 20, 25, 30	10	[15]
BTV-1	*C. bolitinos*	field	10, 15, 18, 23.5, 25, 30	varies	[14]
BTV-1	*C. imicola*	field	10, 15, 18, 23.5, 25, 30	varies	[14]
BTV-9	*C. sonorensis*	colony	12, 15, 20, 25, 30	25	new data; see text
BTV-10	*C. sonorensis*	colony	15, 20, 25, 30	10	[15]
BTV-16	*C. sonorensis*	colony	15, 20, 25, 30	10	[15]
EHDV-1	*C. sonorensis*	colony	15, 20, 25, 30	10	[15]

*AHSV - African horse sickness virus; BTV - bluetongue virus; EHDV - epizootic haemorrhagic disease virus; number indicates serotype.
†number of midges sampled at each time point.
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where $T_{\min}$ is the threshold temperature for virus replication and α reflects the rate of replication above the threshold (with $1/\alpha$ giving the number of degree-days above the threshold temperature required for a vector to complete the EIP).

In the laboratory experiments each midge tested can be in one of three states: (1) infected and has completed the EIP; (2) infected, but has yet to complete the EIP; or (3) uninfected. The probabilities that a midge tested t days post infection when infected at age a and reared at temperature T is in each of these states can be written as,

$$\left.\begin{aligned} q_{taT}^{(1)} &= \varphi \exp\left(-\int_a^{a+t} \mu_I(a',T)\,\mathrm{d}a'\right) \int_0^t f(u;v(T),k)\,\mathrm{d}u, \\ q_{taT}^{(2)} &= \varphi \exp\left(-\int_a^{a+t} \mu_I(a',T)\,\mathrm{d}a'\right) \int_t^{\infty} f(u;v(T),k)\,\mathrm{d}u, \\ q_{taT}^{(3)} &= (1-\varphi) \exp\left(-\int_a^{a+t} \mu_S(a',T)\,\mathrm{d}a'\right), \end{aligned}\right\} \quad (3)$$

where φ is the probability of becoming infected, f is the probability density function for the gamma-distributed EIP (see equation (1)) and $\mu_I(a,T)$ and $\mu_S(a,T)$ are the age- and temperature-dependent mortality rates for infected (I) and uninfected (S) vectors, respectively. The probability that a midge tested t days after infection when reared at temperature T has completed the EIP is given by,

$$p_{tT} = \frac{\sum_a q_{taT}^{(1)} N_a}{\sum_j \sum_a q_{taT}^{(j)} N_a}, \quad (4)$$

where N_a is the number of *Culicoides* of age a at the time of infection and the $q_{taT}^{(j)}$ s are defined in equation (3). Assuming that (i) all *Culicoides* are the same age at infection; and (ii) there is no differential mortality between infected and uninfected vectors (i.e. $\mu_I(a,T) = \mu_S(a,T)$), this probability, (4), simplifies to,

$$p_{tT} = \varphi \int_0^t f(u;v(T),k)\,\mathrm{d}u. \quad (5)$$

We note that the same expression would also be obtained if the vector mortality rates are independent of age (and there is no differential mortality).

Parameter estimation

Model parameters were estimated in a Bayesian framework, which has two components: a likelihood function and a joint prior distribution for the parameters. The number of midges with a fully disseminated infection is drawn from a binomial distribution with the number of midges sampled and the probability that a sampled midge has completed the EIP (given by equation (5)), as parameters. Hence, up to a constant of proportionality, the likelihood for the data is given by,

$$L(\varphi,\alpha,T_{\min},k) = \prod_t \prod_T p_{tT}^{I_{tT}} (1-p_{tT})^{N_{tT}-I_{tT}}, \quad (6)$$

where I_{tT} is the number of midges with a fully disseminated infection and N_{tT} is the number of midges sampled on day t after being reared at temperature T. Non-informative priors were used for the parameters: Uniform(0,1) for φ; diffuse Normal for α and $T_{\min}$; and diffuse exponential for k. The priors were assumed to be independent.

The joint posterior density for the parameters is proportional to,

$$p(\varphi,\alpha,T_{\min},k) \propto L(\varphi,\alpha,T_{\min},k)\pi(\varphi,\alpha,T_{\min},k), \quad (7)$$

where L is the likelihood, (6), and $\pi(\varphi,\alpha,T_{\min},k)$ is the joint prior distribution for the parameters. Samples from the joint posterior distribution were generated using Markov chain-Monte Carlo (MCMC) methods, more specifically, a random-walk Metropolis algorithm [28]. For each data-set five chains of 50,000 iterations were run (with the preceding 10,000 iterations discarded to allow for burn-in of the chain) and were subsequently thinned (by selecting every fiftieth iteration) to reduce correlation amongst the samples. Convergence of the chains was monitored using various measures implemented in the "Convergence Diagnostics and Output Analysis" (CODA) package [29] in R [30]. Model fit was assessed by posterior predictive χ^2 tests [31].

Results

Laboratory determination of EIP for BTV-9 (Kosovo) in *C. sonorensis*

The time to first transmissible infection of *C. sonorensis* by the strain of BTV used was three days at 30°C, four days at 25°C, five days at 20°C, and 20 days at 15°C (Figure 1A, C, E, G). No transmissible infections were recorded up to 40 days of testing at 12°C.

Temperature-dependent model of the EIP

The probability of infection (φ) ranged from 0.04 (BTV-1 in *C. imicola*) to 0.91 (EHDV-1 in *C. sonorensis*) (Table 2). The virus

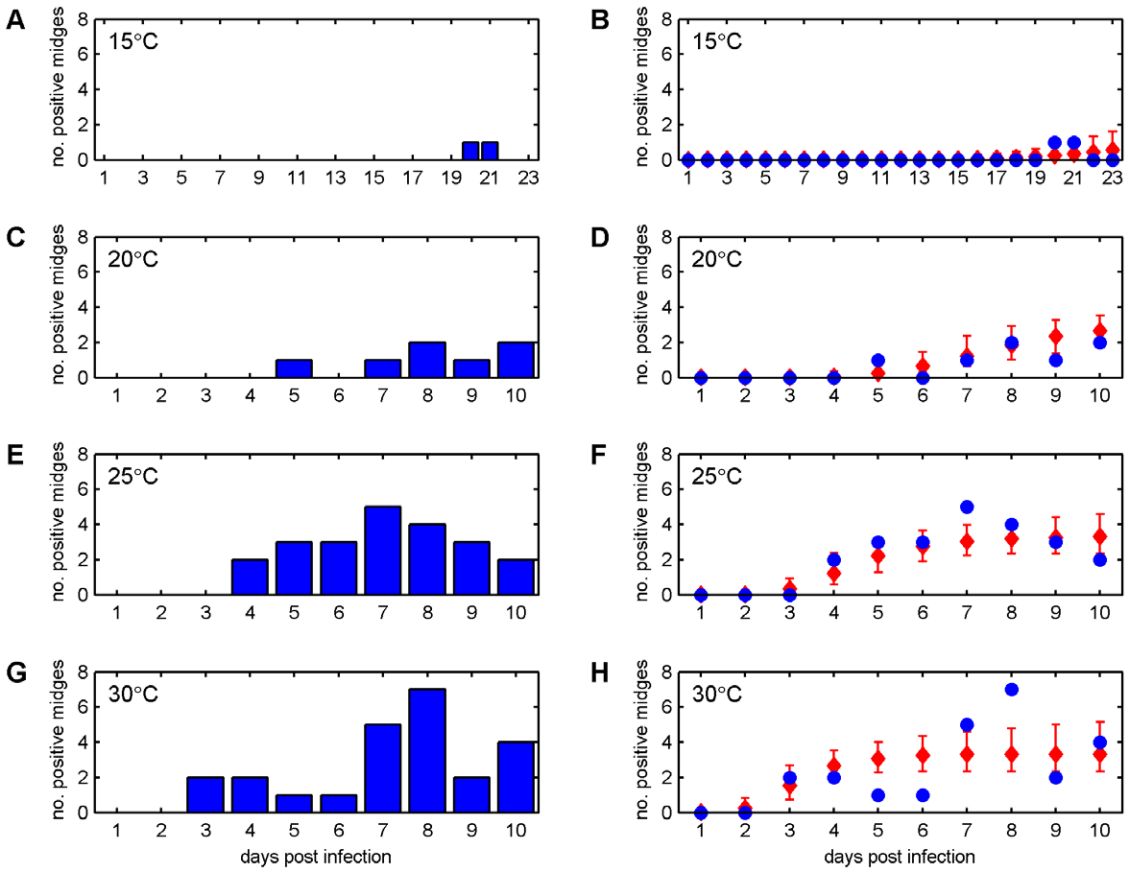

Figure 1. Observed and expected number of *Culicoides sonorensis* with a fully disseminated BTV-9 infection when reared at different temperatures. (A,C,E,G) Number (out of 25) of *C. sonorensis* with a fully disseminated infection. (B,D,F,H) Observed (blue circles) and expected (posterior median (red diamonds) and 95% credible interval (error bars)) number of *C. sonorensis* with a fully disseminated infection. Results are shown for midges reared at (A,B) 15°C, (C,D) 20°C, (E,F) 25°C, or (G,H) 30°C; those for midges reared at 12°C (no midges with a fully disseminated infection) are not shown.
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replication rate (α) was similar for all viruses and vectors except EHDV-1, for which the estimate was much higher (Figure 2A; Table 2). The threshold temperature (T_{min}) was between 11.4°C and 13.3°C for the BTV and AHSV isolates, but was markedly higher (19.5°C) for EHDV-1 (Figure 2B; Table 2). Finally, estimates for the scale parameter (k) were often very high (Table 2), but were significantly ($P<0.05$) different from one for all orbiviruses in the study.

Formal comparison of the parameter estimates for the different orbiviruses/vectors (by sampling from the joint posterior distributions) showed that those for the probability of infection (φ) were similar ($P>0.05$) for all the BTV isolates (9, 10 and 16) tested in *C. sonorensis*, but were significantly ($P<0.01$) different for the other orbiviruses/vector species tested. The virus replication rate (α) for EHDV-1 in *C. sonorensis* was significantly ($P<0.001$) higher than for the other orbiviruses/vector species (which did not differ from one another; $P>0.05$). Finally, the threshold temperature (T_{min}) was significantly ($P<0.001$) higher for EHDV-1 in *C. sonorensis* compared with the other orbiviruses/vector species tested and for BTV-9 in *C. sonorensis* and BTV-1 in *C. bolitinos* compared with BTV-16 in *C. sonorensis* ($P<0.01$).

Posterior predictive checking indicated that the model provided an adequate fit to the data for all orbiviruses, except BTV-1 in *C. bolitinos*. A comparison of the observed and expected values for BTV-9 is shown in Figure 1B, D, F, H; those for the previously published data are shown in supporting information (Figure S1 for data from [15]; Figure S2 for data from [14]).

Discussion

The results of our analyses highlight differences in vector competence for different orbivirus strains and vector species (Table 2). However, the rate of virus replication (approximately 0.017-0.021 per degree-day) and temperature (11-13°C) required for replication were broadly consistent across different orbivirus strains and vector species, with the exception of EHDV-1 (Figure 2; Table 2). The original data produced for EHDV-1 were extremely variable across the temperature range tested [15], however, and require confirmation in future studies.

Comparison of the estimates for the virus replication rate (α) and threshold temperature (T_{min}) derived in the present study (Table 2) with those derived previously for AHSV-4, BTV-10, BTV-16 and EHDV-1 [15] indicate that the earlier methods substantially underestimate both α and T_{min} (Table S1). Moreover, using the estimates for α and T_{min} obtained in [15] (with φ and k estimated independently by maximum likelihood methods using equation (6)) shows a poor fit of the model to data for all four viruses (χ^2 goodness-of-fit tests: $P<0.05$). In particular, there is a tendency to overestimate the number of positive midges at lower temperatures and underestimate the number of positive midges at higher temperatures when using the earlier parameters (Figure S1).

Table 2. Posterior mean, median and 95% credible limits (CL) for parameters in the temperature-dependent model of the extrinsic incubation period for different orbiviruses and vector species.

orbivirus	vector species	probability of infection (φ)				virus replication rate (α)			
		mean	median	95% CL		mean	median	95% CL	
				lower	upper			lower	upper
AHSV-4	*C. sonorensis*	0.52	0.52	0.45	0.59	0.017	0.017	0.014	0.020
BTV-1	*C. bolitinos*	0.61	0.61	0.58	0.64	0.019	0.019	0.018	0.020
BTV-1	*C. imicola*	0.04	0.04	0.03	0.07	0.016	0.016	0.010	0.023
BTV-9	*C. sonorensis*	0.14	0.13	0.09	0.20	0.019	0.019	0.013	0.026
BTV-10	*C. sonorensis*	0.12	0.12	0.08	0.18	0.018	0.018	0.010	0.029
BTV-16	*C. sonorensis*	0.16	0.15	0.12	0.20	0.021	0.020	0.016	0.028
EHDV-1	*C. sonorensis*	0.92	0.92	0.88	0.96	0.084	0.084	0.068	0.105
		threshold temperature (T_{min})				scale parameter (k)			
		mean	median	95% CL		mean	median	95% CL	
				lower	upper			lower	upper
AHSV-4	*C. sonorensis*	12.6	12.7	11.6	13.3	16.7	14.4	6.8	37.7
BTV-1	*C. bolitinos*	13.2	13.2	12.9	13.5	7.8	7.7	5.9	10.1
BTV-1	*C. imicola*	12.7	12.5	10.6	15.5	90.9	66.5	2.7	330.3
BTV-9	*C. sonorensis*	13.3	13.3	12.7	14.1	19.5	14.3	3.5	64.5
BTV-10	*C. sonorensis*	12.9	13.0	11.0	14.0	68.9	46.4	5.0	256.5
BTV-16	*C. sonorensis*	11.4	11.6	9.3	12.6	146.8	116.2	18.9	433.5
EHDV-1	*C. sonorensis*	19.5	19.5	19.3	19.6	6.9	6.6	3.4	12.2

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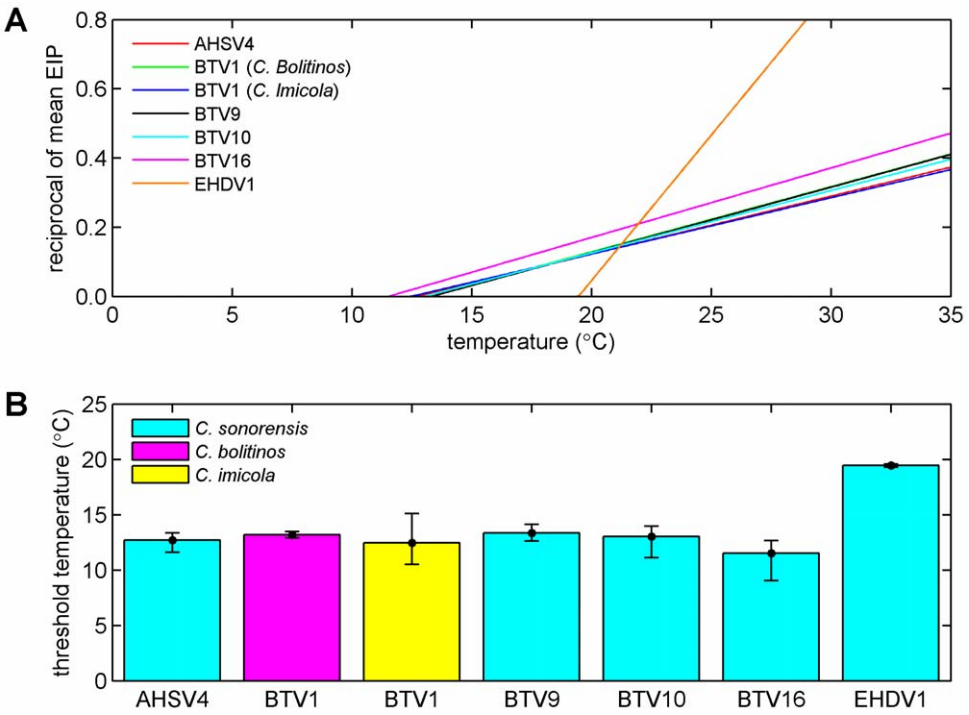

Figure 2. Temperature dependence of the extrinsic incubation period for six orbiviruses. (A) Temperature dependence of the reciprocal of the mean EIP (equation (2) using posterior median estimates for α and T_{min}). Virus (and serotype) is indicated by the line colour (see legend). (B) Posterior median (bars) and 95% credible limits (error bars) for the threshold temperature for virus replication. Vector species is indicated by the bar colour (see legend).
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To explore the reasons for this inaccuracy, we simulated replicated data-sets for each of the four orbiviruses using the model given by equations (1)–(3) and the parameter estimates in Table 2. Applying the methods used in [15] to the simulated data produced similar estimates to those obtained by [15] from the experimental data. By contrast, if the methods used by [15] were applied to the simulated data, but only those for infected midges (which have or have not completed the EIP), they recover the parameter estimates obtained in the present study. This indicates that the earlier methods are limited because they fail to distinguish between infected vectors which have yet to complete the EIP and uninfected vectors.

Two assumptions were made when analysing the data: (i) there is no differential mortality between infected and uninfected vectors; and (ii) there is no age-dependent mortality (or, equivalently, all midges were the same age when infected). Differential mortality has not been studied for *Culicoides* spp. infected with orbiviruses, though there is evidence for increased mortality in mosquito vectors infected with a number of arboviruses (for example, chikungunya virus in *Aedes albopictus* [32]; eastern equine encephalomyelitis virus in *Culiseta melanura* [33]; or Rift Valley Fever virus in *Culex pipiens* [34]). Analysis of the modelling approach, (3)–(4), shows that estimates for the EIP parameters (α, $T_{\min}$ and k) will be unaffected if there is differential mortality, but the probability of infection (φ) will be underestimated. Regarding the second assumption (reviewed recently by [35]), there is some evidence for age-dependent mortality in *C. sonorensis*, but this appears to vary with geographic origin [36]. For the analyses of EIP data obtained from colony-derived *Culicoides* (Table 1), age-dependent mortality is unlikely to have an impact on the parameter estimates, because the midges used in the experiments had all emerged two to three days prior to infection and so were similar ages at infection. This could, however, be an issue for the data obtained from experiments using field-caught vectors (Table 1), where this is unlikely to the case, and may help account for the relatively poor fit of the model to data on the EIP of BTV-1 in *C. bolitinos*.

By constraining the scale parameter (k) to be an integer, it is straight-forward to implement the EIP as series of exponential distributions [37], which can be readily incorporated in orbivirus transmission models [2,38]. In this case, the appropriate choice for the scale parameter is the nearest integer to the value given in Table 2, while the other parameters remain unchanged. Importantly, our results show that the scale parameter (k) differs significantly from one and, hence, the commonly-made assumption of an exponential distribution for the EIP [3,4] is not appropriate for orbiviruses, meaning that other approaches to modelling their EIP should be used [2].

Experimental techniques for the laboratory investigation of the EIP of orbiviruses in *Culicoides*, and the competence of *Culicoides* for orbiviruses, have improved in recent years due the development of rapid detection methodologies (e.g. [39]) which facilitate high-throughput screening of the large numbers of uninfected *Culicoides* generated by vector competence or EIP studies [40,41]. Studies have also shifted from using a hamster-derived cell line for detection purposes (BHK-21) that is prone to variation in sensitivity across lines, orbivirus strains and users, towards the *C. sonorensis*-derived KC cell line which has greater epidemiological relevance to orbivirus studies [27,42]. However, methods for the analysis of the resulting data have not significantly improved during the same period. The method described here represents a substantial improvement upon previous studies, correctly reflecting the experimental design and allowing the rigorous comparison of differences in replication rate amongst orbiviruses and vector species.

Experimental limitations remain in the laboratory determination of the EIP of orbiviruses in *Culicoides* (see also [16]). Chief among these is the lack of a rapid technique to directly determine the degree of orbivirus dissemination within infected *Culicoides* and, hence, provide a realistic view of the probability of transmission of virus to the host [43]. In mosquitoes, salivary glands can be excised relatively straightforwardly and tested for virus [44], or potentially infected individuals can be stimulated to produce saliva which can subsequently be screened [45]. These techniques have been trialled for *Culicoides* [46] but the small size of the subjects makes processing the large numbers of individuals required for both EIP and vector competence studies prohibitively laborious. Instead, most recent studies have inferred transmissibility indirectly using the relationship between the whole-body titre of infected *C. sonorensis* and the presence of virus in the saliva of individuals (as estimated by [26]), or in blood from meals taken by *C. sonorensis* through a membrane based system [25].

The only study to directly trace dissemination in *C. sonorensis* over time, using an immunohistochemistry assay, found that in competent individuals held at $24\pm1^{\circ}$C virus disseminated rapidly from through the midgut and was present in the salivary glands by day five post-infection [26]. This contrasted with a previous study that hypothesised from profiles of virus replication recorded in pools of *C. sonorensis* held at 23°C that the period of replication and dissemination within the haemocoel, following entry through the midgut, was substantial and lasted up to 6 days [47]. Hence, while the presence of virus in saliva of *Culicoides* has been inferred for the current study as representing transmissible infections it is currently unclear what degree of replication in secondary organs is required to allow onwards transmission to the host. Further studies taking into account additional factors that may enhance transmission efficacy [48] and utilising appropriate live hosts, have the potential to clarify this relationship, particularly when combined with advances in technologies for visualising virus dissemination within the insect vector.

A second experimental limitation to current studies is that detailed investigations require the use of large numbers of colony-bred *Culicoides*. This has led to reliance upon the use of *C. sonorensis* lines due to the difficulty of colonising alternative African or European vector species. The successful production of blood feeding methods for field populations of *C. imicola* in Southern Africa has raised the potential of laboratory colonisation for this species [49]. Research on methodologies to produce sustainable colonies of those northern European species that have been implicated in BTV transmission have, however, been limited to date and require further examination.

Despite these qualifications, the results presented here remain the most robust to date for the orbivirus-*Culicoides* system. In terms of practical application, the results presented here could be used to inform the timing of livestock movement restrictions which are used to control the spread of BTV and other orbiviruses. Current EU legislation already permits the movement of unvaccinated animals during periods of minimal *Culicoides* activity, as measured using light trap networks (see [50] for a review). The earliest date on which it is possible for transmission from vectors to hosts to resume could be estimated from temperature-EIP relationships such as the ones described in the present study using thermal time accumulation models [51] similar to those used already to forecast crop growth [52] and the development of insect pests [53], provided the temperature of the environment in which the vectors rest after feeding is known. Such an approach has already been suggested for West Nile virus in the United States [54]. A strategy based upon a combined dataset of seasonal activity of *Culicoides* and a temperature-derived "transmission-free period" could

potentially allow safe animal movements for longer periods and with more warning of revocation. This could lead to substantial economic benefits for the livestock industry, but is dependent upon accurate quantitative understanding of the relationship between temperature and EIP, emphasising the need for such relationships to be examined in detail using the listed recommendations to improve both the accuracy of laboratory studies and subsequent analyses.

Supporting Information

Figure S1 Observed and expected number of *Culicoides sonorensis* with a fully disseminated infection when reared at different temperatures. Each figure shows the observed (blue circles) and expected (posterior median (red diamonds) and 95% credible interval (error bars)) number of positive *C. sonorensis* infected with different orbiviruses: African horse sickness virus (AHSV); bluetongue virus (BTV); and epizootic haemorrhagic disease virus (EHDV) (the number indicates serotype). The data were extracted from [15]. The cyan squares show the expected number of positive midges using the estimates for α and $T_{\min}$ obtained by [15] with φ and k estimated independently by maximum likelihood methods.
(TIF)

Figure S2 Observed and expected number of *Culicoides bolitinos* or *Culicoides imicola* with a fully disseminated infection when reared at different temperatures. Each figure shows the observed (blue circles) and expected (posterior median and 95% credible interval: red diamonds and error bars) number of positive *C. bolitinos* or *C. imicola* infected with bluetongue virus serotype 1. The data were extracted from [14].
(TIF)

Table S1 Previous estimates for the temperature dependence of the extrinsic incubation period of different orbiviruses in *Culicoides sonorensis*.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SC GV PM. Performed the experiments: SC JB EV GV. Analyzed the data: SG AW SC. Wrote the paper: SC AW GV SG PM.

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Appendix 5

Sushila Maan, Narender S. Maan, Kyriaki Nomikou, **Eva Veronesi**, Katarzyna Bachanek-Bankowska, Manjunatha N. Belaganahalli, Houssam Attoui and Peter P.C. Mertens. **2011**.

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Abstract

Bluetongue virus is the "type" species of the genus *Orbivirus,* family *Reoviridae.* Twenty four distinct bluetongue virus (BTV) serotypes have been recognized for decades, any of which is thought to be capable of causing "bluetongue" (BT), an insect-borne disease of ruminants. However, two further BTV serotypes, BTV-25 (Toggenburg orbivirus, from Switzerland) and BTV-26 (from Kuwait) have recently been identified in goats and sheep, respectively. The BTV genome is composed of ten segments of linear dsRNA, encoding 7 virus-structural proteins (VP1 to VP7) and four distinct non-structural (NS) proteins (NS1 to NS4). We report the entire BTV-26 genome sequence (isolate KUW2010/02) and comparisons to other orbiviruses. Highest identity levels were consistently detected with other BTV strains, identifying KUW2010/02 as BTV. The outer-core protein and major BTV serogroup-specific antigen "VP7" showed 98% aa sequence identity with BTV-25, indicating a common ancestry. However, higher level of variation in the nucleotide sequence of Seg-7 (81.2% identity) suggests strong conservation pressures on the protein of these two strains, and that they diverged a long time ago. Comparisons of Seg-2, encoding major outer-capsid component and cell-attachment protein "VP2" identified KUW2010/02 as 26th BTV, within a 12th Seg-2 nucleotype [nucleotype L]. Comparisons of Seg-6, encoding the smaller outer capsid protein VP5, also showed levels of nt/aa variation consistent with identification of KUW2010/02 as BTV-26 (within a 9th Seg-6 nucleotype - nucleotype I). Sequence data for Seg-2 of KUW2010/02 were used to design four sets of oligonucleotide primers for use in BTV-26, type-specific RT-PCR assays. Analyses of other more conserved genome segments placed KUW2010/02 and BTV-25/SWI2008/01 closer to each other than to other "eastern" or "western" BTV strains, but as representatives of two novel and distinct geographic groups (topotypes). Our analyses indicate that all of the BTV genome segments have evolved under strong purifying selection.

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Introduction

Bluetongue virus (BTV) is the type-species of the genus *Orbivirus*, the largest of fifteen genera within the family *Reoviridae* [1,2]. BTV can infect ruminants, camelids, and occasionally large carnivores [3,4,5]. The virus is transmitted by biting midges (*Culicoides* spp.) in which it also replicates. It can sometimes also be transmitted either via an oral route, or vertically in sheep and cattle [6,7]. Clinical signs of BTV infection are often confined to sheep or white-tailed deer and are usually more severe in naïve populations [8,9]. Cattle and goats are largely (although not exclusively) asymptomatic and can be considered as reservoir hosts [10]. However, the 'western' strain of BTV-8 which recently spread across Europe also caused some clinical signs and a low level of mortality in cattle [9].

BTV virus particles are approximately 80 nm in diameter, icosahedral in symmetry and are composed of three concentric protein layers, surrounding a genome composed of 10 linear segments of double-stranded (ds) RNA [11,12]. BTV genome segments range in size from 3954 to 822 bp (total of 19.2 kbp) and are identified as 'segment 1 to 10' (Seg-1 to Seg-10) in order of decreasing molecular weight and/or increasing electrophoretic mobility in 1% agarose gels [1]. Twenty five serotypes of BTV have previously been recognised, the identity of which is determined by the specificity of reactions between neutralising antibodies (generated during infection of the mammalian host) and components of the outer-capsid (VP2 and VP5) [1,13,14].

Sequencing studies and phylogenetic comparisons show that Seg-2 and to a lesser extent Seg-6 (encoding outer-capsid proteins VP2 and VP5 respectively) are the most variable components of the BTV genome, varying in a manner that correlates with virus serotype [15,16,17]. Sequences of BTV Seg-2 can be divided into 25 distinct clades that correlate exactly with the virus serotype and can be used to identify virus type in sequencing studies or RT-PCR assays. Seg-2 sequences for different serotypes can also grouped into a smaller number of nucleotypes (nucleotypes A to L), which correlate with serological cross-reactions that have been detected between the different BTV types [15,16,18].

Structural -proteins, VP3[T2] and VP7[T13] (encoded by Seg-3 and Seg-7) form the innermost 'sub-core', and 'core-surface' layers of the virus-particle, respectively, and are more highly conserved between BTV serotypes than the outer-capsid proteins [1,6,16,17,19,20,21]. VP7 has been identified as the major *Orbivirus* species / serogroup specific antigen [22] and previous phylogenetic comparisons have used Seg-3 sequences to identify members of individual *Orbivirus* species [23,24]. BTV also encodes three other highly conserved enzyme-proteins, which represent minor components of the sub-core particle, including: the RNA dependent RNA polymerase - VP1(Pol); the capping enzyme - VP4(CaP); and the helicase VP6(Hel), encoded by Seg-1, Seg-4 and Seg-9 respectively [25].

Four non-structural BTV proteins have also been identified in BTV-infected cells but are not present in purified virions [11,26,27,28]. The two larger and the smallest non-structural proteins (NS1(TuP), NS2(ViP) and NS4) are highly conserved across different BTV serotypes [29,30]. However, NS3/NS3a can be more variable within some other *Orbivirus* species, representing the second most variable protein of AHSV, after VP2 [31,32].

The entire BTV genome, including both the 'conserved' and more 'variable' segments (represented by Seg-2 and Seg-6), show significant nucleotide-sequence variations that at least partially correlate with the geographic origins of the virus isolate / lineage. This suggests that the emergence of individual BTV serotypes was followed by a significant period of geographic isolation allowing mutations to accumulate, generating geographically distinct virus lineages or 'topotypes' [15,16,17,33].

Since 1998, multiple BTV types have emerged within Europe, events that have been linked to international trade and climate change in the region, raising concerns about possible future threats posed by bluetongue and other related orbiviral diseases [6,7,34,35]. Multiple exotic BTV types have also been identified (during the same period) in the south-eastern USA [36].

During early 2008, an atypical BTV was detected in clinically healthy goats from the Toggenburg region of north eastern Switzerland, using a BTV-specific real-time RT–PCR (rRT-PCR) targeting Seg-10 designed by Orru et al [37]. Sequence analyses show that this novel strain is distinct from members of the 'major' eastern and western BTV topotypes previously identified by Maan et al [16]. Attempts to isolate the virus in cell culture have so far been unsuccessful, making it difficult to confirm its serotype by virus neutralisation tests (VNTs) [38]. However, sera from the infected goats failed to neutralise reference strains of the 24 established BTV serotypes in serum neutralisation tests (SNTs), and together with phylogenetic analyses of Seg-2 nt-sequences, this has identified it as a novel 25th BTV serotype (BTV-25/TOV) [14,16].

In February 2010, sheep and goats in Kuwait showed clinical signs of disease [39]. Analyses of twenty six blood samples from the Abdali region, identified only two positive samples for BTV, one of which was used to isolate an orbivirus (strain KUW2010/02). VNT using antisera against the existing 25 BTV types failed to neutralise this new virus and it has therefore been proposed as a novel 26th BTV serotype [39]. In order to further characterise the isolate and help determine its relationships, the entire genome of KUW2010/02 was sequenced and compared to other orbiviruses, including multiple BTV isolates. The results from these analyses are presented and discussed.

Materials and Methods

Virus isolation, propagation in cell culture

Twenty six EDTA treated blood-samples, five organ-samples (four spleens; one liver) from sheep and goats suspected of infection with BTV, were sent from Kuwait to the OIE reference laboratory for BTV at Institute for Animal Health (IAH) in the UK, during 2010. These samples were taken from naturally infected animals in the field, by qualified veterinarians, as part of normal diagnostic testing procedures in Kuwait and did not therefore require Ethics Committee approval.

Washed blood was inoculated intravenously into embryonated chicken eggs (ECE) (UK Home Office licence number PPL 70/6213), and then passaged twice in BHK-21 clone 13 cells (European Collection of Animal cell Cultures [ECACC – 84100501]) (E1/BHK2). Only one blood sample from Animal No. 374 (which is stored as 'KUW2010/01' in the 'dsRNA virus reference collection' (dsRNA-VRC) [40] was used successfully to isolate virus (isolate number KUW2010/02). The virus was also passaged twice in Vero cells (ECACC – 84113001) (E1/BHK1/Vero2) until cytopathic effects (CPE) were observed (isolate KUW2010/03).

Serology

Virus isolates KUW2010/02 and KUW2010/03 were tested by indirect antigen-sandwich ELISA [41] and virus titre was calculated using the Spearman-Karber formula and expressed as $TCID_{50}$/ml.

Virus neutralisation tests (VNT) were performed on KUW2010/02 (using antisera to BTV-1 to BTV-25) to identify the BTV- type in this isolate. A standard 'constant serum - varying virus' method was used (with appropriate controls) in a micro titre plates [42]. A 'neutralisation' result showing at least 100 fold reduction in virus titre by a 'type-specific' reference antiserum, as compared to reactions containing a negative control serum, is regarded as evidence of a specific reaction (same serotype).

Extraction of RNA and identification of BTV

RNA was extracted from EDTA treated blood using QIAamp Viral RNA Mini Kit (Qiagen) or Universal BioRobot (Qiagen), as per manufacturer's protocol, for use in serogroup and serotype specific real-time RT-PCRs (rRT-PCRs) described earlier [37]. RNA was also purified from infected cells (KUW2010/02 or KUW2010/03) for full-length cDNA synthesis using TRIzol (Invitrogen) [15,43]. Viral RNA extracted from KUW2010/02 was analysed by agarose gel electrophoresis (AGE) and used for sequencing the entire virus genome.

RT-PCR for full-length cDNA amplification and sequencing

Full length cDNA copies of BTV genome segments were synthesised and amplified, after 'anchor spacer–ligation' as described by Maan et al [44]. 'Phased primers' were used to sequence near-terminal regions of Seg-2 and Seg-6, while primers corresponding to conserved 5′ and 3′ terminal sequences were used to sequence the remaining genome segments [16]. The individual cDNA amplicons, purified using a 'GFX^{TM} PCR DNA and gel band purification kit' (Amersham Pharmacia Biotech, Inc), were sequenced on a 3730 capillary sequencer (Applied Biosystems). Sequence data for the entire genome of KUW2010/02 have been submitted to GenBank (Table 1).

Phylogenetic and positive selection analysis

Consensus sequences for individual genome segments were assembled and analyzed using SeqMan Software (DNAStar Inc.) then aligned with data for other BTV strains from GenBank [16], using CLUSTAL X [45] and MAFFT ver 5 [46].

RevTrans 1.4 Server (http://www.cbs.dtu.dk/services/RevTrans/) [47] was also used for each set of DNA sequences. This

Table 1. Characteristics of dsRNA genome segments (cDNA copy) and proteins of the bluetongue virus serotype 26 Kuwait (KUW2010/02).

Genome segment (Size: bp)	ORFs bp (including stop codon)	Number of Amino acids/Protein nomenclature	Accession numbers	Highest Percentage Identity (nt/aa) with other BTV topotypes				
				Closest Eastern Strain (nt identity)	Closest Eastern strain(s) (aa identity)	Closest Western Strain (nt identity)	Closest Western Strain (aa identity)	BTV-25 (SWI2008/01) [Accession No.] (nt/aa identity)
1 (3944)	12–3920	1302/VP1 (Pol)	JN255156	RSArrrr/16 (BTV-16) (75.3%)	RSArrrr/16 (BTV-16) BUL1999/01 (BTV-9) (87.3%)	RSAvvvv/02 (BTV-2) (75.8%)	NET2007/01 (BTV-8) (87.8%)	[Ac. No. GQ982522] 75.6%/ 86.8%
2 (2929)	20–2893	957/VP2	HM590642	BTV-23 Australia [Ac. no. U04200] (48.7%)	ISA1991/02 (BTV-23) (38.4%)	BTV-17 USA [Ac. no. AY636073] (62.0%)	BTV-10 USA [Ac. no. U06780] (58.1%)	[Ac. No. EU839840] 63.9%/ 61.5%
3 (2773)	18–2723	901/VP3 (T2)	HM590643	GRE2001/07 (BTV-1) (75.8%)	RSArrrr/16 (BTV-16) ISR2001/18 (BTV-16) (88.9%)	RSArrrr/09 (BTV-9) (76.4%)	RSArrrr/02 (BTV-2) (88.6%)	[Ac. No. GQ982523] 76.6%/ 88.9%
4 (1982)	9–1943	644/VP4 (Cap)	JN255157	BTV-12 Taiwan [Ac. no. GU390661] (73.9%)	GRE1999/13 (BTV-16) (81.3%)	BTV-10 USA [Ac. no. Y00421] (74.8%)	USA2003/05 (BTV-5) (81.5%)	[Ac. No. GQ982524] 74.5%/ 82.3%
5 (1758)	35–1693	552/NS1 (TuP)	JN255158	SER2001/01 (BTV-9) (73.6%)	BUL1999/01 (BTV-9) (80.4%)	NIG1982/07 (BTV-8) (74.4%)	TAT1990/02 (BTV-17) (81.2%)	[Ac. No. EU839841] 72.5%/ 78.8%
6 (1629)	30–1601	523/VP5	JN255159	AUS1979/02 (BTV-21) (69%)	SER2001/01 (BTV-9) (72.4%)	RSArrrr/24 (BTV-24) (73%)	RSArrrr/11 (BTV-11) (79.3%)	[Ac. No. EU839842] 69.1%/ 73.4%
7 (1157)	18–1067	349/VP7 (T13)	HM590644	BTV-23 India [Ac. no. AJ277802] (78.6%)	BTV-2 China [Ac. no. AF172826] (94.3%)	BTV-1 France [Ac. no. FJ437558] (79.2%)	USA2003/05 (BTV-5) (94.8%)	[Ac. No. EU839843] 81.2%/ 97.7%
8 (1121)	19–1080	353/NS2 (ViP)	JN255160	AUS–––/01 (BTV-1) (70.8%)	BTV-12 Taiwan [Ac. no. GU390665] (69.4%)	RSArrrr/01 (BTV-1) (71.6%)	TAT1990/02 (BTV-17) (70%)	[Ac. No. EU839844] 69.3%/ 70.9%
9 (1070)	16–1026 185–415	336/VP6 (Hel) 77/ NS4	JN255161	BOS2002/02 (BTV-9) (70.9%)	BOS2002/02 (BTV-9) (64.8%)	BTV-10 USA [Ac. no. U55780] (71.2%)	RSAvvv3/04 (BTV-4) (63.8%)	[Ac. No. EU839845] 73.7%/ 75%
10 (822)	20–709 59–709	229/NS3 216 NS3a	JN255162	BTV-2 Taiwan [Ac. no. AY493685] (81.3%)	GRE1998/01 (BTV-9) (86%)	BTV-6 South Africa [Ac. no. GQ506497] (80.5%)	NET2007/01 (BTV-8) (86%)	[Ac. No. EU839846] 82.6%/ 89.5%

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translates nt data, aligns the resulting peptide sequences, then uses this 'scaffold' to construct multiple DNA alignments that maintain reading frame integrity. A best fit model (selected using the Akaike Information Criterion [AIC] and Bayesian Information Criterion [BIC]) of nucleotide substitution was selected for the coding region of each genome segment [48], for maximum likelihood analysis using Mega 5, as well for positive selection analysis (see below). AIC and BIC selected different nucleotide substitution models for various genome segments of BTV: GTR+G+I (Seg-9); GTR+G (Seg-10); TN93+G+I (Seg-1, -2, -6 and -8); T92+G+I (Seg-3, -4 and -5) and T92+G (Seg-7). Phylogenetic trees from each genome segment were also constructed using neighbour-joining methods and distance matrices, generated by p distance determination algorithm in MEGA version 5 software (500 bootstrap replicates) [48].

The sequence data set for each genome segment was checked for evidence of recombination, using the Genetic Algorithm for Recombination Detection (GARD), www.datamonkey.org/GARD, [49] and Recombination Detection Program (RDP), http://darwin.uvigo.es/rdp/rdp.html [50]. The Tajima D test of neutrality, implemented in MEGA5, was used to assess selection.

For each of these aligned data sets we estimated the rates of non-synonymous and synonymous changes (Positive selection analysis) at each site, using likelihood-based methods as implemented in the on-line Datamonkey server (http://www.datamonkey.org; [49]. These analyses used: i) a conservative single likelihood ancestor-counting (SLAC) method, which is related to that of Suzuki–Gojobori [51] and ii) a fixed-effects likelihood (FEL) method. Both SLAC and FEL methods were used to calculate the global ratio of non-synonymous substitutions per non-synonymous site (dN) to synonymous substitutions per synonymous site (dS) (expressed as dN/dS) using default (estimated) option. A dN/dS ratio $\sim$1 signifies neutral evolution; dN/dS $>$1 positive/diversifying selection; and dN/dS $<$1 negative/purifying selection.

Development of conventional, gel-based BTV-26 specific RT-PCR assays

RNA from KUW2010/02 was tested by conventional and real-time RT-PCR assays using primers directed against Seg-2 of different BTV serotypes (conventional primers – [52]; real-time assays available from Laboratoire Service International [LSI], Lissieu, France). cDNA amplicons from the conventional assays were analysed by AGE.

Four sets of primers targeting Seg-2 of KUW2010/02 (Ac. No. HM590642) were designed after comparison to multiple BTV isolates of different serotypes [15,16]. Each primer-pair was evaluated using RNA extracted from BTV-26 (KUW2010/02 and KUW2010/03); BTV-25 (SWI2008/01) (Nucleotype K); and BTV-4, 10, 11, 17, 20 and 24 (the most closely related heterologous serotypes - Nucleotype A) [15]. Primer footprints were compared (*in silico*) with Seg-2 sequences from other BTV serotypes, to confirm type specificity.

Results

Thirty one blood and tissue samples from Kuwait were tested using four different real-time RT-PCR (rRT-PCR) assays designed to detect BTV RNA [39]. All of the samples gave negative results with assays targeting either Seg-1 [53], or Seg-1 and 5 [54]. However, two blood samples (from animals 364 and 374 [KUW2010/01]) were positive for BTV when tested with an assay targeting Seg-9 (Maan et al – in preparation) and Seg-10 (designed by Orru et al [37]. RNA extracted from KUW2010/01 was also tested by 'type-specific' rRT-PCRs targeting Seg-2 (LSI), for European BTV serotypes (BTV-1, 2, 4, 6, 8, 9, 11 and 16), with negative results.

Virus was successfully isolated from one blood-sample (KUW2010/01) and grown in BHK cells (isolate KUW2010/02) or Vero cells (isolate KUW2010/03) [39]. KUW2010/02 and KUW2010/03 were both confirmed as BTV using an indirect sandwich ELISA to detect BTV-VP7 [41] with OD_{490} values $>$0.15 [39]. KUW2010/02 was also tested in virus neutralisation tests (VNT), using reference guinea pig immune-sera against BTV-1 to BTV-24, as well as BTV +ve antiserum from goats previously infected with BTV-25 (SWI2008/01). None of these antisera caused significant levels of neutralisation, indicating that KUW2010/02 does not belong to previously recognised BTV serotypes (BTV-1 to 25) [39].

Viral RNA extracted from KUW2010/02 was analysed by AGE, and as previously reported [39] generated a migration pattern (electropherotype) typical of BTV, or a closely related orbivirus. RNA from KUW2010/02 was also tested by type-specific, real-time RT-PCR assays, targeting Seg-2 of BTV serotypes 1 to 25 (LSI), with uniformly negative results.

Sequence and phylogenetic analysis of the genome segments of KUW2010/02

Full-length cDNA copies of KUW2010/02 genome segments were synthesised and both strands of each genome segment were analysed so that consensus sequences could be unambiguously determined. All genome segments have the conserved RNA termini (+ve 5′-GUUAAA.....ACUUAC-3′) that are typical of bluetongue virus [55].

BLAST analysis of sequences from KUW2010/02 consistently showed highest levels of sequence identity to homologous genome segments of other BTV isolates. Results of phylogenetic analyses using CLUSTAL X and MAFFT alignments, neighbour-joining and maximum likelihood tree construction, all located the genome segments of KUW2010/02 within the BTV serogroup/species, confirming the results of BLAST analyses (Figures 1, 2 and 3 – see below). The use of neighbour-joining (p distance) and maximum likelihood methods did not alter the clustering or phylogenetic relationships of any KUW2010/02 genome segment to a great extent.

Segment 1. Comparisons of Seg-1 from KUW2010/02 with other BTVs, showed that it is conserved at 3944 base pairs (bp), encoding the 1302 amino acid (aa) of VP1[Pol] (Table 1). The sequences of Seg-1/VP1[Pol] are also highly 'conserved', with overall nt/aa identity levels of 74.6%/86.0% to 75.8%/87.8% to other BTV isolates and a maximum of 67.5%/68.8% to members of other *Orbivirus* species (EHDV-1/NIG1967/01 and EHDV-6/AUS1981/07, respectively), confirming its identification as a novel BTV isolate. However, KUW2010/02 does not cluster within either of the major BTV topotypes previously identified, showing similarly low maximum nt/aa identity levels to members of both 'eastern' and 'western' topotypes (Table 1) and to BTV-25 (SWI2008/01), which represents a distinct (western) topotype [16]. These data indicate that BTV-26 (KUW2010/02) also represents a further distinct (eastern) group/topotype.

Segment 2. Seg-2 of KUW2010/02 is 2929 bp long, encoding 957 aa of VP2 (Table 1) showing nt/aa identity levels of 42.8%/28.3% to 63.9%/61.5% to previously recognised BTV serotypes. As previously reported [39] these low values identify KUW2010/02 as a novel 26th type within a distinct 12th Seg-2 nucleotype 'L' [15,16,39]. The sizes of Seg-2 and VP2 of KUW2010/02 show differences in length when compared to other BTV serotypes. Seg-2 of KUW2010/02 showed slightly higher levels of identity to the SWI2008/01 strain of BTV-25,

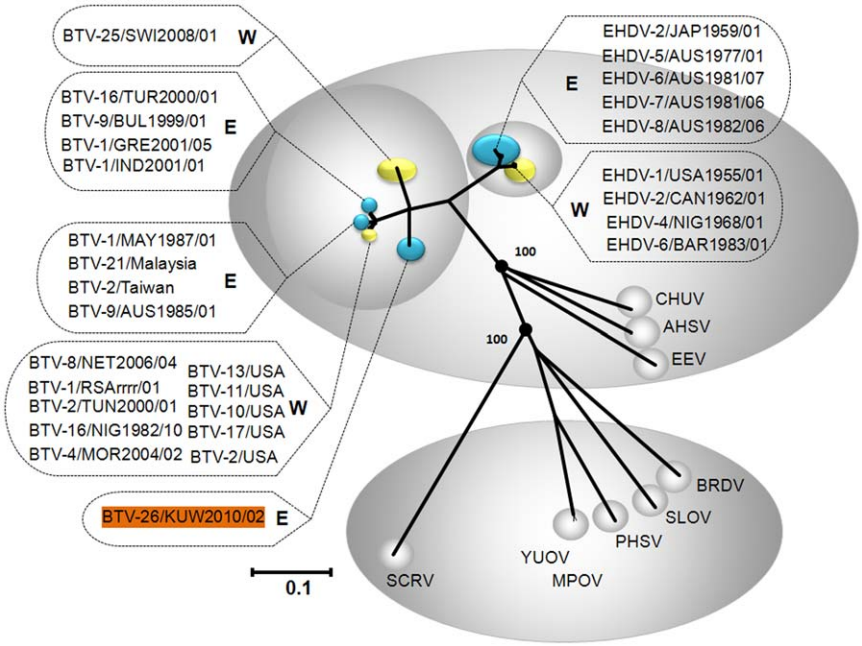

Figure 1. Neighbour-joining tree showing relationships between VP3[T2] of KUW2010/02 with other orbiviruses. KUW2010/02 showed up to 76.6%/88.9% nt/aa identity in Seg-3/VP3[T2] with other BTV strains confirming that it is an isolate of BTV. Accession numbers and further detail of the sequence and viruses used are included in Table 1. The tree was constructed using distance matrices, generated using the p-distance determination algorithm in MEGA 5 (500 bootstrap replicates) [48]. The trees shown in Figures 2 and 3 were drawn using same parameters. The scale bar indicates the number of substitutions per site. Values at the nodes indicate bootstrap confidence. Epizootic haemorrhagic disease virus (EHDV), Bluetongue virus (BTV), Equine encephalosis virus (EEV), African horse sickness virus (AHSV), Chuzan virus (CHUV), St. Croix River virus (SCRV), Yunnan orbivirus (YUOV), Middle point orbivirus (MPOV), Peruvian horsesickness virus (PHSV), Broadhaven virus (BRDV), Stretch Lagoon Orbivirus (SLOV). Eastern and western isolates of EHDV and BTV are shown in blue and yellow respectively. Seg-3 accession numbers used for comparative analyses: AM745079, AM745029, AM745039, AM745049, AM745059, AM744979, AM744999, AM745019, AM745069, NC_005989, AF021236, FJ183386, M87875, NC_012755, NC_007749, NC_007657, EF591620, NC_005998, DQ186827, DQ186797, DQ186822, DQ186811, DQ186816, AF529047, AY493688, DQ186790, AM498052, DQ186792, DQ186826, DQ186819, DQ186817, L19969, L19968, NC 006014, AF017281, L19967.
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than to reference strains of BTV-10 and BTV-17 from the USA (Table 1).

Segment 3. Seg-3 of KUW2010/02 is 2773 bp long, encoding 901 aa of the highly conserved BTV sub-core-shell protein, VP3(T2) (Table 1), showing 73.7%/87.6% to 76.6%/88.9% nt/aa identity with other BTVs. Although lengths are otherwise conserved, the 3′ NCR of KUW2010/02 Seg-3 is one nucleotide longer than other BTV isolates that have been analysed (N = >80). Closest relationships were detected with 'eastern' strains of BTV-16 from Israel, and 'western' reference strains of BTV-2 and 9 (Table 1). Similar levels of identity were also detected with BTV-25 (SWI2008/01).

In comparisons with the T2 gene of multiple other *Orbivirus* species, KUW2010/02 showed a maximum of 69.9%/77.5% nt/aa identity with EHDV (EHDV-4/NIG1968/01). From previous studies these identity levels confirm KUW2010/02 as an isolate of BTV [16,23]. None of the previously characterised BTV strains show much closer relationships to KUW2010/02 in Seg-3 (Figure 1, Table 1 and 2), indicating that it does not cluster within the previously recognised topotypes [16,17,21] and therefore (as indicated for Seg-1) represents a further distinct (eastern) group/topotype.

Segment 4. Seg-4 of BTV is 1982 nt in length, encoding 644 aa of the highly conserved VP4 capping enzyme protein (CaP) (Table 1), showing nt/aa identity levels of 72.3%/79.3% to 74.8%/82.3% with other BTVs. Although lengths are otherwise conserved, the 3′ NCR of KUW2010/02 Seg-4 is one nucleotide longer than the other BTV isolates analysed (N = >70). Highest overall identity levels were detected between KUW2010/02 and BTV-10 USA (western topotype), BTV-16 Greece (eastern topotype) and to BTV-25 (SWI2008/01), consistent with membership of a distinct (eastern) group/topotype (Table 1).

Segment 5. Seg-5 of BTV-26 KUW2010/02 is 1758 nt long, encoding 552 aa of the highly conserved NS1 tubule protein (TuP). However, these lengths showed considerable variation, particularly in the 3′ NCR (Table 1), when compared to other BTV isolates (N = >85). Seg-5/NS1[TuP] of KUW2010/02 shows nt/aa identity levels of 72.5%/79.3% to 74.4%/81.2% with other BTVs, and closest relationships to BTV-9 from Serbia and Bulgaria (eastern toptotype) and BTV-8 from Nigeria and BTV-17 from Trinidad and Tobago (western topotype). Similar nt/aa identity levels were also detected with BTV-20 Australia (Ac. No. X56735) and BTV-25 (SWI2008/01), which individually form additional 'far eastern' and 'western' topotypes, again indicating that KUW2010/02 represents another discrete eastern group/topotype.

Segment 6. Seg-6 of BTV-26 KUW2010/02 is 1629 nt long, encoding the 523 aa of VP5, the smaller of the two outer-capsid components and second most variable of the BTV proteins (Table 1). This is the smallest Seg-6/VP5 that has been recorded for any BTV (by 9 nucleotides and 3 amino acids), showing nt/aa identity levels of only 57.1%/41.4% to 73.0%/79.3% to previously recognised BTV serotypes. Closest relationships were detected with BTV-21 Australia, BTV-9 Serbia (eastern topotype),

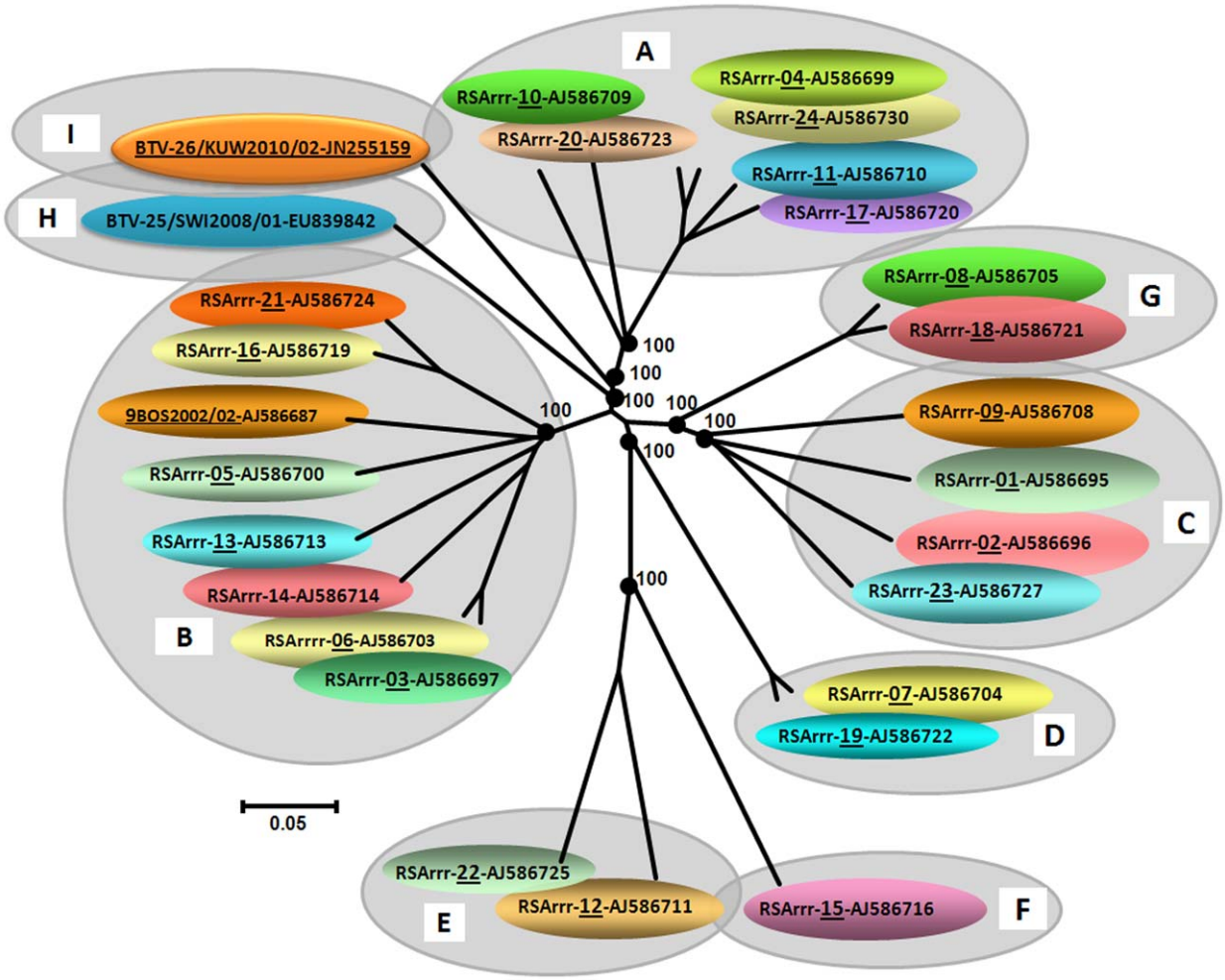

Figure 2. Neighbour-joining tree showing relationships between Seg-6 from KUW2010/02 with the twenty five reference strains of different BTV serotypes. The eight evolutionary branching points are indicated by black dots on the tree (along with their bootstrap values), dividing the sequences into nine 'Seg-6 nucleotypes' designated 'A–I'. In previous studies, eight Seg-6 nucleotypes were identified. Members of the same nucleotype show >76% nt identity in Seg-6, while members of different nucleotypes show <76% nt identity [16]. However the analyses of BTV-26 (KUW2010/02) described here indicate that it forms a new 9th Seg-6 nucleotype (I), as it shows a maximum of 73.0%/79.3% nt/aa identity with previously existing BTV serotypes. Seg-6 accession numbers used for comparative analyses: AJ586695 - AJ586699, AJ586700, AJ586703 - AJ586711, AJ586713, AJ586714, AJ586716, AJ586719, AJ586720 - AJ586725, AJ586727, AJ586730, EU839842.
doi:10.1371/journal.pone.0026147.g002

reference strains of BTV-11, BTV-24 (western toptotype) and with BTV-25 (SWI2008/01) (Table 1). As seen for Seg-2/VP2, these identity levels also support the identification of KUW2010/02 as a distinct 26th virus 'type', within a novel 9th Seg-6 nucleotype 'I' [16] (Figure 2).

Segment 7. Seg-7 of KUW2010/02 is 1157 bp long, encoding 349 aa of the major BTV serogroup-specific antigen and core surface protein - VP7 (Table 1). These lengths are similar to those of some other but not all previously characterised BTV isolates (N = >100). Sequence comparisons of Seg-7/VP7[T13] confirmed KUW2010/02 as an isolate of BTV, with identity levels ranging from 69.2%/80.8% to 81.2%/97.7% to other isolates, and closest relationships with BTV-25 (Figure 3). Close relationships were also detected with BTV-23 from India and BTV-2 from China (eastern topotype); BTV-1 from France and BTV-5 from the USA (western topotype) (Table 1).

Segment 8. Seg-8 of KUW2010/02 is 1121 bp long encoding the 353 aa of the viral inclusion body (VIB) matrix protein - NS2 (Table 1). This Seg-8/NS2 is four nucleotides and one aa shorter than any BTV strain previously analysed (N = >98). Seg-8/NS2[ViP] of KUW2010/02 show nt/aa identity levels of 67.6%/65.9% to 71.6%/70.9% with other BTVs, and closest relationships to BTV-1 Australia, BTV-12 Taiwan (eastern topotype); reference strain of BTV-1 and BTV-17 from Trinidad and Tobago (western topotype) and BTV-25/TOV (Table 1). As observed with the other conserved segments, KUW2010/02 represents a second distinct 'eastern' topotype.

Segment 9. Seg-9 of the KUW2010/02 is 1070 bp, encodes VP6, a minor core protein and the helicase enzyme (Hel) of 336 aa in length, as well as NS4 (from an out of frame ORF), which is 77 aa in length [27] (Table 1). This is 18 nt/6 aa longer than Seg-9/VP6 of 'eastern' BTV strains (N = 51) and 21 nt/7 aa longer than Seg-9/VP6 of 'western' strains previously characterised (N = 102). Seg-9/VP6[Hel] from KUW2010/02, shows identity levels that range from 64.3%/53.6% to 73.7%/75.0% to other BTV isolates, with closest relationships to BTV-9 from Bosnia (eastern topotype), BTV-10 USA, the reference strain of BTV-3 (western topotype) and BTV-25 (Table 1). As with the other genome segments

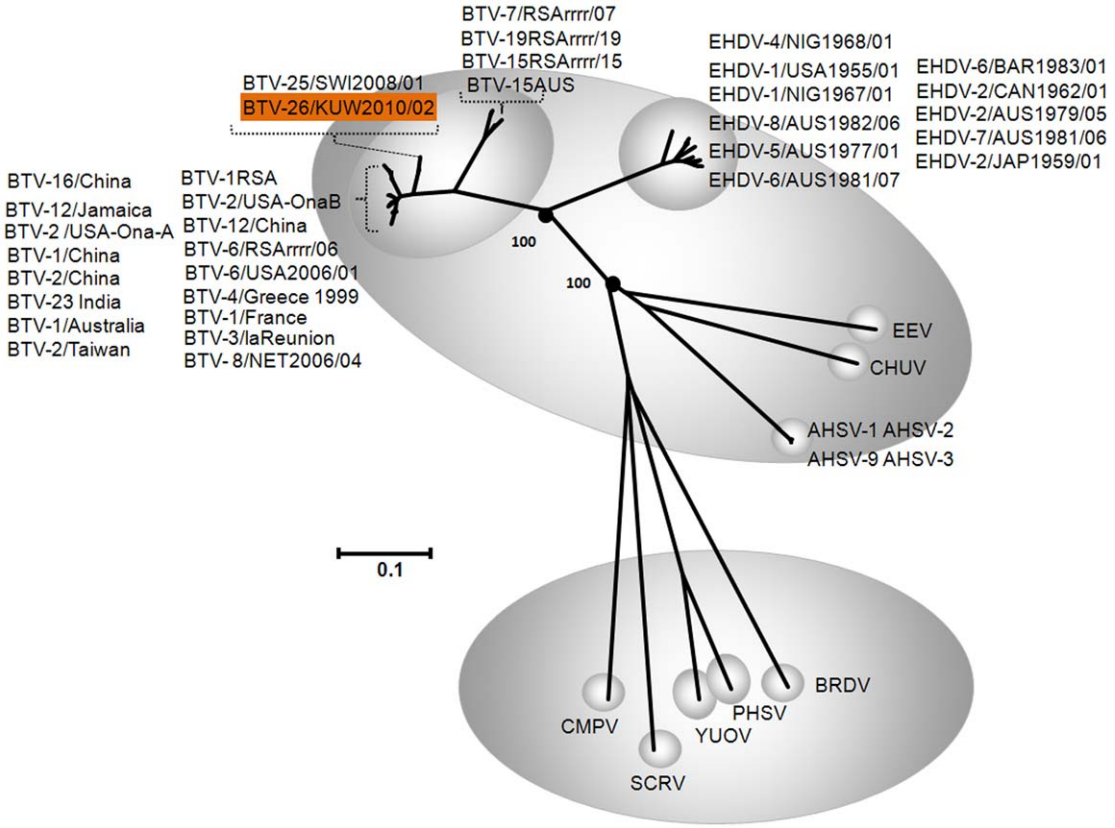

Figure 3. Neighbour-joining tree showing relationships between VP7[T13] from KUW2010/02 with other orbiviruses. KUW2010/02 showed between 69.2%/80.8% to 81.2%/97.7% nt/aa identity in Seg-7/VP7[T13] to other BTV isolates, confirming its identity as a member of the *Bluetongue virus* species. Accession numbers and further detail of the sequence and viruses used are included in Table 1. Epizootic haemorrhagic disease virus (EHDV), Bluetongue virus (BTV), Equine encephalosis virus (EEV), African horse sickness virus (AHSV), Chuzan virus (CHUV), St. Croix River virus (SCRV), Yunnan orbivirus (YUOV), Peruvian horsesickness virus (PHSV), Broadhaven virus (BRDV) and California mosquito pool virus (CMPV). Seg-7 accession numbers used for comparative analyses: AM745023, AM744983, AM745013, AM745063, AM745033, AM745043, AM745073, AM745003, AM744993, AM745053, AM745083, FJ183391, AY078469, FJ183371, HM035361, HM035392, AF545433, M87876, NC 007754, NC 007663, NC 006004, ACF22097, AY485667, AM498057, FJ437558, AY841352, GQ506542, GQ506502, AF172829, AF188660, X53740, AY493692, M63417, AJ277802, AF172826, AF172825, AF188674, AF188673, AF172831, EU839843, L11724, DQ465027, DQ465028, DQ465026.
doi:10.1371/journal.pone.0026147.g003

analysed, these data indicate that BTV-26 KUW2010/02 represents a further distinct 'eastern' topotype. As with other BTVs, NS4 of KUW2010/02 is also highly conserved.

Three consecutive amino acid sequence repeats were identified within VP6 of KUW2010/02. These repeats which are shown in Figure 4, are located between codon positions 205 – 232 and may explain why VP6 of KUW2010/02 is so long. These repeats are outside the NS4 region (nt 185 – 415). Interestingly each repeat was found to align best with the protein sequence immediately upstream of it within in VP6. However, the repeated sequences are not fully identical. This suggests sequence duplication has been followed by some 'evolution' of the parental and the daughter repeated sequences [56,57].

Segment 10. Seg-10 of KUW2010/02 is 822 bp long and codes for two, related non-structural proteins, NS3 (229 aa) and NS3a (216 aa) (Table 1). These lengths are identical to other BTV strains analysed (N = >95). Seg-10/NS3 of KUW2010/02 show nt/aa identity levels of 76.5%/84.3% to 82.6%/89.5% with other

Table 2. Summary of percentage sequence identities for Seg-3/VP3[T2] between the eastern viruses, western viruses, BTV-25/SWI2008/01 and KUW2010/02.

	Major eastern topotype		Major western topotype		BTV-25 (SWI2008/01)		BTV-26(KUW2010/02)	
Major eastern topotype	>89.8	***>98.1***						
Major western topotype	79.3–82.4	***96.9–99.3***	>87.5	***>97.7***				
BTV-25 (SWI2008/01)	74.9–76.7	***88.0–88.8***	75.0–76.1	***88.5–89.5***	ID	***ID***		
BTV-26 (KUW2010/02)	74.6–75.8	***87.9–88.9***	75.0–76.4	***87.6–88.6***	76.6	***88.9***	ID	***ID***

Both nucleotide (nt) and amino acid (aa-bold italics) identities are presented.
doi:10.1371/journal.pone.0026147.t002

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205 KK---N-RPAD 211
213 KKGGEREKAPE 223
     **    + +**+

213 KKGGEREKA 221
225 RS-NQREKA 232
     *+    ****
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Figure 4. Examples of contiguous repeats found in the aa sequence of KUW2010/02 VP6. Evidence was detected for repeated contiguous aa sequences in VP6 of KUW2010/02. The aa positions, as indicated, are between residues 205 to 232. The region 213 to 223 is shown as the target sequence, with matching repeats 205–211 (upstream) and 225–232 (downstream), shown in the upper and lower lines respectively. + similar residue: * identical residue.
doi:10.1371/journal.pone.0026147.g004

BTVs, and closest relationships with BTV-2 Taiwan and BTV-9 Greece (eastern strains), BTV-6 South Africa and BTV-8 Netherlands (western strains), and BTV-25 (SWI2008/01) (Table 1). In a pattern similar to other segments these data for Seg-10 of BTV-26 KUW2010/02 indicate that it represents the first isolate of a distinct 'eastern' topotype.

Positive/negative selection analysis

The Tajima D test of neutrality, implemented in MEGA5, was used to assess selection. The expected value for populations that conform to a standard neutral model for selection is zero [57]. However the D values obtained for Seg-1 to Seg-10 reject the 'null hypothesis' for neutral selection of the BTV segments.

Recombination can adversely affect the power and accuracy of phylogenetic reconstruction [58] and may result in higher rates of false positives in maximum likelihood tests for positive selection [59].

No evidence of recombination was detected in Seg-4 to Seg-10 using GARD and RDP, whereas in Seg-1, Seg-2 and Seg-3 both programs showed evidence of one breakpoint, although the results were inconclusive. Positive selection analysis was performed separately for each genome segment of BTV. The SLAC and FEL methods did not identify any sites in the majority of the BTV genome segments with evidence of significant positive selection at the p, 0.1 level. However, in the Seg-9, 18 codon sites (5, 38, 63, 70, 72, 87, 90, 92, 93, 97, 98, 112, 119, 125, 126, 128, 131 and 132) ($p = 0.09$) were identified by the FEL method as being influenced by positive selection, where as SLAC only identified 6 codon sites (72, 87, 97, 125, 126 and 131) that were positively selected ($p = 0.079$). The majority of these positively selected codons are in the NS4 ORF in Seg-9 (between codons 60–138) [27].

Positive selection analyses were also performed separately for the eastern and western lineages of Seg-9. Seg-9 sequences of KUW2010/02 and SWI2008/01 were used in both eastern as well as western analysis, as each of them makes a separate eastern and western cluster respectively. The SLAC method did not identify any site in the eastern lineage which gave evidence of positive selection significant at the p 0.1 level. However, codons 75 and 97 in the western lineage, were identified by the SLAC method as influenced by positive selection ($p = 0.65$). FEL methods identified 6 (codon 5, 63, 64, 69, 70, 103) and 15 (5, 55, 63, 72, 75, 87, 92, 97, 98, 112, 119, 123, 125, 128, 131) positively selected sites in the eastern and western lineage respectively significant at the p 0.1 level.

The global estimate of dN/dS by SLAC method for Seg-1 to Seg-10 were 0.036, 0.132, 0.018, 0.0516, 0.051, 0.048, 0.022, 0.087, 0.242, 0.047, respectively (using estimated (default) option where dN/dS is estimated from the data), indicating purifying or strong purifying selection. A high number of negatively selected codons were also identified (significant at the $p = 0.1$ level) in each genome segment with SLAC and FEL (data not shown) suggesting that all BTV genes evolved under negative/purifying selection.

RT-PCR assays

Sequence data generated for Seg-2 of KUW2010/02, and comparisons to other BTV types, were used to design four sets of oligonucleotide primers for conventional RT-PCR assays (Table 3). All four primer-pairs (1 to 4) worked well, generating products of the expected sizes from the original blood sample (KUW2010/01) and both passage levels of BTV-26 (KUW2010/02 and KU2010/03) (Figure 5). Although other combinations of these forward and reverse primers also appeared to be effective, they were not widely

Table 3. Primers for amplification of Seg-2 from BTV-26 in RT-PCR assays.

Primer Pair	Primer Name*	Primer Sequence (5′-3′)	Position on genome Seg-2 (nt)	Predicted Product size (bp)
Pair 1	BTV-26/S2/176-196F BTV-26/S2/1289-1268R	TCTAAGCAAGGGATTATCGAT TAACTTCCTCATCAACTGAGAT	176–196 1289–1268	1113[†]
Pair 2	BTV-26/S2/1267-1286F BTV-26/S2/1849-1831R	TATCTCAGTTGATGAGGAAG GCATATATCCCTTTCACCT	1267–1286 1849–1831	582[†]
Pair 3	BTV-26/S2/1819-1839F BTV-26/S2/2213-2194R	ACATTACGCTAGAGGTGAAAG GATCACGAATCACCTCGACG	1819–1839 2213–2194	394[‡]
Pair 4	BTV-26/S2/286-303F BTV-26/S2/1943-1919R	GATGAGGACAGCACGGAA GACCGTGGTGATATTGTGGATCAAG	286–303 1943–1919	1657[‡]

*Individual primers are identified by the BTV serotype (e.g. BTV-26) followed by the letter S and number 2 (to indicate Seg-2), then a number to indicate the relative nucleotide position of the primer within VP2 gene, followed by F or R to indicate forward or reverse orientation.
[†]Primer-pairs 1 and 2 also generated very faint but near right sized bands from Seg-2 of certain serotypes within nucleotype 'A' (BTV-4, 10, 17, 20 and 24 – Primer-pair 1; BTV-4, 10 and 17 – Primer-pair 2), the most closely related nucleotype/serotypes to BTV-26 and therefore they cannot be regarded as BTV-26 specific.
[‡]Primer-pairs 3 and 4 although generated multiple bands of low intensity with some serotypes in the nucleotype 'A' but none of them was of right size, so these two sets can be regarded as BTV-26 specific.
doi:10.1371/journal.pone.0026147.t003

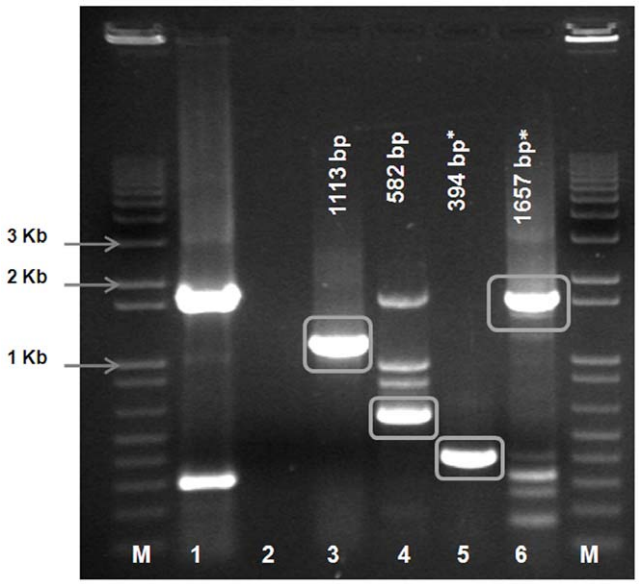

Figure 5. Electrophoretic analysis of cDNA products generated from Seg-2 of BTV-26 (KUW2010/02) using primer-pairs designed from the homologous sequence. PCR amplicons were generated from Seg-2 of BTV-26, isolate KUW2010/02 using primer-pairs 1 – 4 - Table 3 (lanes 3 to 6 respectively). Primer-pairs 3 and 4 are BTV-26 specific, while primer-pairs 1 and 2 also amplifies certain other serotypes in Seg-2 nucleotype 'A'. Lane 1 is a positive control using RNA from BTV-6/RSArrrr/06, with primer-pair BTV-6/2/301F & BTV-6/2/790R – 1631 bp [16]. Lane 2 is a negative water control. Lane M: 1 kb marker.
doi:10.1371/journal.pone.0026147.g005

evaluated (data not shown). Primer-pairs 1 to 4 were also tested with reference strains of the most closely related heterologous serotypes (BTV-4, 10, 11, 17, 20 and 24, belonging to nucleotype 'A' [15]. Primer-pairs 1 and 2 generated faint bands that were near to the 'predicted' size, with some strains from nucleotype 'A' (Primer-pair 1 with BTV-4, 10, 17, 20 and 24; Primer-pair 2 with BTV-4, 10 and 17). Therefore primer-pairs 1 and 2 are not considered to be entirely BTV-26 specific. However, any cDNA amplicons generated can be sequenced using the same primer sets, helping to identify both the virus strain and its relationships to other isolates.

Although primer-pairs 3 and 4 generated multiple low intensity bands with RNA from some of the serotypes in the nucleotype 'A', none of these products were the correct size, and these two sets are therefore regarded as BTV-26 specific. In each case unambiguous identification of BTV- 26 can also be achieved by sequencing and phylogenetic comparisons to the cDNA generated (as described here). KUW2010/02 represents a reference stain for the novel BTV serotype 26.

Discussion

Many viruses with RNA genomes can rapidly adapt to and exploit rapidly changing global landscapes and local environments. Genetic variation (mutation, recombination, and reassortment) and environmental factors (including trade, ecosystem, communal, and health care factors) can play important roles in the selection, emergence and evolution of different viruses. This paper presents full genome sequence data for the reference strain of a novel BTV serotype (BTV-26) for further comparative studies.

Blood/tissue and serum samples, from sheep and goats in Kuwait showing clinical signs of disease (suspected BTV infection), were sent from the Diagnostic Laboratory Centre (PAAF-Kuwait) to IAH-UK for testing. Most of the serum samples were positive for BTV specific antibodies, indicating previous BTV infection (there is no BTV vaccination policy in Kuwait). However, BTV-RNA was only detected in two sheep blood samples (animals 364 and 374) using a BTV-Seg-9 (Maan et al – in preparation) and BTV-Seg-10 specific rRT-PCR assay (designed by Orru et al [37] that had previously also been used to detect BTV-25 in Switzerland [14], suggesting that the ongoing and more widespread clinical signs observed were not due to a current BTV infection. However, BTV Seg-1, or Seg-1 and 5 specific assays [53,54] failed to detect RNA of the Kuwait virus, indicating that it was an unusual or atypical BTV strain. Experimental infections of sheep with KUW2010/02 caused only mild clinical disease (Chris Oura – Personal communication). Further diagnostic, pathogenesis and insect transmission studies will add to our knowledge of this novel BTV serotype/topotype.

Identification of KUW2010/02 as an isolate of BTV

When analysed by AGE, KUW2010/02 generated a migration pattern typical of a BTV isolate, indicating that it is a member of this virus species [39].

Earlier studies of Seg-3/VP2[T2] from different orbiviruses, initially showed >91% aa identity within the same species/serogroup [24]. However, subsequent studies that included multiple BTV isolates from different geographic regions (topotypes), detected as little as 74.9% nt/87.8% aa identity in Seg-3/VP3 [16].

In the study presented here, Seg-3/VP3 of KUW2010/02 showed up to 76.6% nt/88.9% aa identity with other BTV strains (Table 1), confirming that it belongs to the same virus species. However, 73.7% nt identity with BTV-15 Australia [Ac. No. AY322427] and 87.6% aa identity with BTV-2 USA [Ac. No. L19967], have further reduced the lower identity limits detected within the species. Similar results were obtained with the other conserved genome segments (Seg-1, -4, -5, -7, -8, -9 and -10), in each case confirming KUW2010/02 as an isolate of BTV, although again slightly reducing the lower limit of identity detected between BTV isolates in Seg-1, -4, -8 and -9.

VP7[T13] is the major serogroup-specific antigen of BTV and related orbiviruses [22,60]. KUW2010/02 not only gave high-level positive results in a BTV-specific antigen-ELISA targeting VP7 [41], it also showed up to 97.7% nt/aa identity to another BTV strain (SWI2008/01), consistent with its identity as a member of the *Bluetongue virus* species.

Identification of KUW2010/02 as BTV-26

Neutralisation assays demonstrated that none of the antisera against BTV-1 to BTV-25, caused significant levels of neutralisation, indicating that KUW2010/02 belongs to a novel 26th BTV type [39].

Seg-2/VP2 of KUW2010/02 showed a maximum of 63.9% nt and 61.5%/aa identity with BTV-25 (SWI2008/01). These levels are significantly lower than previously detected within a single BTV serotype (minimum levels of 68.4% nt/72.6% aa – [16]), confirming the identification of KUW2010/02 as BTV-26, and as a 12th Seg-2 nucleotype (L). However, these values also slightly increase the maximum level of identity detected between different BTV serotypes (previous maximum of 61.4% nt/59.5% aa).

We have designed two initial pairs of conventional primers for the amplification and detection of Seg-2 from KUW2010/02, which do not amplify Seg-2 of other BTV serotypes and in this respect can be regarded as 'type specific'. However, we recognise that other strains of BTV-26 may be isolated in future, which have

sequence differences in the footprints of these initial primer sets. Seg-2 of any such viruses will need to be sequenced, so that these 'type-specific' primers can be redesigned, maintaining their specificity.

Seg-6/VP5, which can also influence BTV serotype [13], showed a maximum of 73.0% nt/79.3% aa identity between KUW2010/02 and any other BTV type (Table 1), indicating that it belongs to a distinct and 9th Seg-6 nucleotype (I) (Figure 2) [16,17]. This is again consistent with its identification as BTV-26. The lowest similarity detected in Seg-6/VP5 between KUW2010/02 and other BTV serotypes was 57.1% nt and 41.4% aa, slightly above levels previously detected between BTV-25 strain SWI2008/01 and other BTV isolates (at 56.9% nt and 40.8% aa) [16].

We therefore propose KUW2010/02 as the reference strain for this novel serotype, with the Seg-2 specific primer-pairs designed for conventional RT-PCR assays and sequencing studies, providing initial diagnostic tools for BTV-26.

Identification of KUW2010/02 as a novel major topotype

Most BTV isolates can be divided between two major 'eastern' or 'western' topotypes (reflecting their geographic origins) then into a number of further geographic subgroups based on phylogenetic analyses of their genome segments [16,17]. Viruses within the same major-topotype showed >87.5% nt identity in Seg-3, while a maximum of 82.4% nt identity was detected between the major eastern and western groups/topotypes (Table 2). The data presented here show a maximum of 75.8%, 76.4% or 76.6% nt identity between Seg-3 of KUW2010/02 and eastern topotype, western topotype or BTV-25 respectively. These data indicate that KUW2010/02 and BTV-25 (SWI2008/01) represent two new and distinct groups of Seg-3 sequences [16], and may therefore represent additional 'major' topotypes (Figure 1, Table 1 and 2).

Evolutionary selection of BTV sequences

All of the BTV genes, including those coding for VP2 – VP7 and NS1 - NS3, appear to have evolved under purifying selection (sometimes strongly so), evidenced by the dN/dS values of <1. Relatively high dN/dS value suggested of that protein translated from Seg-2 and Seg-9 might be targets for periodic positive selection. The majority of positively selected codons in Seg-9, fall in the ORF for NS4 (60–138 aa) [27], indicating significant functional constraints. Importantly, VP2 determines BTV serotype and is the most variable segment in the viral genome, whereas Seg-9 encodes the viral helicase VP6 and NS4, which is highly conserved in all BTVs. The role of NS4 has yet to be identified, although bioinformatic analyses indicate that it contains coiled-coils and is related to proteins that bind nucleic acids, or are associated with lipids or membranes. The results obtained for Seg-2, 3, 6 and 10 are consistent with previous conclusions [21,61,62]. Lee et al. [63] have also reported similar findings, except for the VP7 gene, which they suggest has a positive or diversifying selection (dN/dS ratios ranged from 1.2 to 5.7 (2.85 ± 2.0; $n = 4$)). In contrast we find using greater numbers of VP7 sequences and a more diverse data set, that negative or purifying selection dominates the evolution of all BTV genes, most likely due to the constraint imposed by the alternate arthropod-vertebrate host transmission cycle. There are reports that some other vector-borne RNA viruses including West Nile virus [64] and Venezuela equine encephalitis virus [65] also evolve under purifying selection [66].

Sequence comparisons of most of the conserved genes and proteins place KUW2010/02 and SWI2008/01 (BTV-25) in additional but distinct geographic groups (representing additional major topotypes of BTV). KUW2010/02 and SWI2008/01 show only 81.2% nt sequence identity in Seg-7, again indicating that they have evolved separately as members of distinct geographic groups (topotypes) a for long period of time. However, a very high level of aa identity (97.7%) was detected in VP7[T13] between KUW2010/02 and SWI2008/01, indicating that they share a common ancestry and suggesting very strong conservation pressures / functional constraints on the sequence of VP7 between these two strains. It is therefore possible for conservation pressures on aa sequence to mask the regional variations between orbivirus topotypes, even though they are still evident as relatively large variations in nt sequence.

The provision of a full genome sequence for the novel BTV serotype (BTV-26) will make it possible to track any further changes, or reassortment events, that occur if BTV-26 continues to persist or spread in the region.

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Author Contributions

Conceived and designed the experiments: SM NSM KN PPCM. Performed the experiments: SM NSM KN EV MNB. Analyzed the data: SM NSM KN HA MNB PPCM. Contributed reagents/materials/analysis tools: SM NSM KN PPCM KB-B MNB HA. Wrote the paper: SM NSM PPCM.

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Appendix 6

Maan, S., S.N Maan, N. Ross-smith, C.A. Batten, E.A. Shaw, S.J. Anthony, A.Samuel, K.E. Darpel, **E. Veronesi**, C.A.L. Oura, K. Singh, K. Nomikou, C. Potgieter, H. Attoui, E. van Rooij, P. van R.K. De Clercq, F. Vandenbussche, S. Zientara, E. Breard, C. Sailleau, M. Beer, B. Hoffman, P.S. Mellor, P.P.C. Mertens. **2008**.

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ABSTRACT

During 2006 the first outbreak of bluetongue ever recorded in northern Europe started in Belgium and the Netherlands, spreading to Luxemburg, Germany and north-east France. The virus overwintered (2006–2007) reappearing during May–June 2007 with greatly increased severity in affected areas, spreading further into Germany and France, reaching Denmark, Switzerland, the Czech Republic and the UK. Infected animals were also imported into Poland, Italy, Spain and the UK. An initial isolate from the Netherlands (NET2006/04) was identified as BTV-8 by RT-PCR assays targeting genome segment 2. The full genome of NET2006/04 was sequenced and compared to selected European isolates, South African vaccine strains and other BTV-8 strains, indicating that it originated in sub-Saharan Africa. Although NET2006/04 showed high levels of nucleotide identity with other 'western' BTV strains, it represents a new introduction and was not derived from the BTV-8 vaccine, although its route of entry into Europe has not been established.

Introduction

'Bluetongue' (BT) is an economically important disease affecting sheep (with case fatality rates that can occasionally exceed 70% Gambles, 1949), some species of deer and to a lesser extent cattle and goats. The bluetongue virus (BTV) is transmitted almost exclusively by adult female hematophagous midges, belonging to certain species of the genus *Culicoides,* and can infect most ruminant or camelid species. BT in sheep is characterised by a loss of condition, muscle degeneration, coronitis, haemorrhages, respiratory distress and swelling of the head and neck, which occasionally leads to cyanosis of the tongue (after which the disease is named). Clinical signs of BT can be particularly severe in naive sheep populations (in areas that are normally free of the disease), or after introduction of an exotic strain into endemic areas (Darpel et al., 2007; MacLachlan 2004; Prasad et al., in press).

A series of BT outbreaks that started in 1998 has spread across much of southern and central Europe, involving eight different virus strains, belonging to five distinct serotypes (BTV-1, 2, 4, 9 and 16). These viruses arrived in Europe via three distinct routes: from the east through Turkey into Greece and Bulgaria; from Algeria or Tunisia into Italy and the eastern Mediterranean islands, or via Morocco into the Iberian Peninsula (Mellor and Wittmann, 2002; Mertens et al., 2007b). The arrival of multiple BTV strains and their persistence in Europe have been linked to the effects of climate change (particularly higher temperatures) and its influence on both the distribution and vector capacity of local *Culicoides* populations (Conte et al., 2003; Purse et al., 2005; Purse and Rogers, in press).

The record temperatures experienced in northern Europe during the summer of 2006 coincided with the first outbreak of bluetongue ever recorded in the region, spreading across the Netherlands, Belgium,

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Luxemburg, much of Germany and into north-east France (Darpel et al., 2007; OIE, 2006a; Toussaint et al., 2006). During the first year of the outbreak morbidity and mortality levels were low, for example it is estimated that Belgium lost less than 100 animals in total (Elbers et al., 2007). However, the virus overwintered successfully (2006–2007) reappearing in the same regions during May–June 2007, then spreading further across Central France and Germany, arriving in Denmark and the East Anglian region of the UK during September/October 2007, then into Switzerland and the Czech Republic. Most significantly the incidence and severity of the disease dramatically increased in the second year with case fatality levels in sheep approaching 50% in some areas. During the late summer it was estimated that Belgium alone lost >15% of its entire sheep population killed by the disease. There were reports of reduced productivity, loss of milk yield, and low levels of mortality in cattle (<1%), as well as clear indications of vertical transmission (particularly in cattle — Zentis, 2008), and abortion/sterility in both cattle and sheep (Hoogendam, 2007; Wilson and Mellor, 2007). Movement of cattle within Europe resulted in the importation of BTV-8 infected animals and outbreaks in Spain and Italy in 2007–2008. Small numbers of infected animals were also imported into Poland, Romania and the UK (including England, Scotland and Northern Ireland). At the end of 2007 this was already the largest and most economically damaging outbreak caused by a single BTV strain on record. Although there are some plans for mass vaccination, it appears likely that the outbreak will continue to spread and increase in intensity during 2008, particularly in newly affected areas.

Bluetongue virus is the type species of the genus *Orbivirus* within the family *Reoviridae* (Mertens et al., 2005). The virus genome is composed of ten segments of linear double stranded RNA (Verwoerd, 1970), which code for ten distinct viral proteins (Mertens et al., 1984, 1987a,b, 2007c). The inner layers forming the 'core' of the BTV capsid contain VP1, VP3, VP4, VP6 and VP7 (encoded by genome segment (Seg) 1, 3, 4, 9 and 7 respectively). These core proteins and two of the non-structural proteins (NS1 and NS2: encoded by Seg-5 and 8 respectively) are thought to be relatively conserved, and are antigenically cross-reactive between different strains and serotypes of BTV. However, cross-hybridisation and sequencing studies have shown that these genome segments can vary in a manner that reflects the geographic origin of the virus strain (topotype) (Gould and Pritchard, 1990; Mertens et al., 1987b; Pritchard et al., 1995, 2004).

Genome segment 10 (encoding non-structural proteins NS3/N3a of BTV) is more variable than the majority of the genome segments encoding the other non-structural or core proteins. The significance of variations in NS3/NS3a is not fully understood, although it is clear that these non-structural proteins do not determine virus serotype. It has been suggested that variations in Seg-10 might relate to transmission of the virus by different insect vector populations and species (Balasuriya et al., 2008; Bonneau et al., 1999; Nikolakaki et al., 2005; Wilson et al., 2007). Seg-7 of BTV, encoding the outer core protein VP7, also shows significant variations (Wilson et al., 2000), despite the role of VP7 as the major serogroup-specific antigen. It has been suggested that these variations could also relate to the insect populations that act as vectors for different virus strains in different geographic areas (Bonneau et al., 2000; Wilson et al., 2000).

The BTV outer capsid proteins VP2 and VP5 (encoded by Seg-2 and Seg-6 respectively) determine the specificity of interactions between the virus particle and the neutralising antibodies generated during infection of the mammalian host (Cowley and Gorman, 1989; Huismans et al., 1985; Mertens et al., 1989). Genome segments 2 and 6 show high levels of sequence variation that correlate with virus serotype (particularly in VP2/segment 2 — Maan et al., 2007a; Mertens et al., 2007g; Singh et al., 2004). However, these genome segments also show variations within each serotype that correlate with the geographic origin of the virus strain (Seg-2 and 6 'topotypes') (Bonneau et al., 1999; Gould and Pritchard 1990; Maan et al., 2007a; Mertens et al., 2007g; Singh et al., 2004; Zhang et al., 1999).

Bluetongue virus was isolated from the blood of sheep showing severe clinical signs of disease in the Netherlands during August 2006 (IAH reference collection number NET2006/04). We report the first identification of this virus as BTV-8, the cause of the northern European BT outbreak in 2006. We also report the complete nucleotide sequence of the virus genome, and a comparison of each genome segment to other European field strains, and the BTV vaccine strains that have been used in the region. Comparisons were also made to representative 'eastern' and 'western' bluetongue viruses from other parts of the world (including other strains of BTV-8), in an attempt to clarify the origins of the outbreak virus.

Results

Virus isolation and propagation in cell culture

Blood samples from sheep showing clinical signs of bluetongue (sample number A83/06-6) were sent from the Netherlands to the Community Reference Laboratory (CRL) at IAH Pirbright. Initial attempts to isolate virus directly into BHK-21 cells failed, even though the sample was positive by ELISA and real-time RT-PCR. No virus (or CPE) was detected when material passaged in embryonated chicken eggs (BTV8-E1) was titrated on BHK cells, although BTV8-E1 was positive for BTV RNA by real-time RT-PCR (CT 18.05). However, supernatant from *Culicoides sonorensis* cell cultures (KC cells), 7 days after inoculation with RBC from sample number A83/06-6, did cause 100% CPE in BHK-21 cells at 4 days post infection. This virus isolate was added to the IAH reference collection as NET2006/04 — (passage level KC1/BHK1 — Mertens et al., 2007d).

Identification and typing of NET2006/04

The northern European virus was identified as BTV by RT-PCR assays targeting Seg-7 (Anthony et al., 2007), generating an amplicon of the expected size (1156 bp) from EDTA treated blood samples, infected egg material and infected cell-culture supernatants (Fig. 1—Panel D). Real-time RT-PCR assays targeting Seg-1 (Shaw et al., 2007) confirmed this result and showed that the virus belongs to a 'western' lineage, indicating that it originated from Africa or America.

Serotype specific RT-PCR assays targeting Seg-2 only generated amplicons of the expected size with the primers for BTV-8 (Mertens et al., 2007b, and in preparation) excluding the other 23 BTV serotypes (Fig. 1—Panels A–C). This represents the first positive identification of BTV-8 in Europe and was confirmed using multiple additional primer pairs and sequencing of Seg-2. Sequence comparisons with Seg-2 of reference strains for the 24 BTV serotypes (Maan et al., 2007a), showed 93.4% nt sequence identity with the BTV-8 reference strain (RSArrrr/08).

Full-length sequence analyses and comparison of the NET2006/04 genome

Genome segments 1 to 10 of NET2006/04 range in size from 3944 (Seg-1) to 822 base pairs (bp) (Seg-10), encoding proteins from 1302 (VP1) to 229/216 amino acids (aa) (NS3/NS3A) respectively. Details of individual genome segments are given in Table 1.

Genome segment 1 of BTV-8 NET2006/04 was compared to available data for other 'western' viruses, and to BTV-2 from Taiwan (Table 1 — supplementary data). In each case Seg-1 is 3944 bp long, encoding the RNA polymerase protein (Pol) of 1302 aa. The 5′ non-coding regions (NCR) is 11 bases long and is 100% conserved, while the 3′ NCR is 24 nucleotides (nt) long and is >91.6% conserved, for the BTV strains analysed (Table 1). The full-length sequence of Seg-1 is also highly 'conserved', showing >78.7% identity overall, with NET2006/04 most closely related (94.5% identity) to BTV-2 from Corsica 2002 (Ac. No. AY154458). The African and American isolates of BTV-2 (L20508); BTV-10 (X12819); BTV-11 (L20445); BTV-13 (L20446); and BTV-17 (L20447)

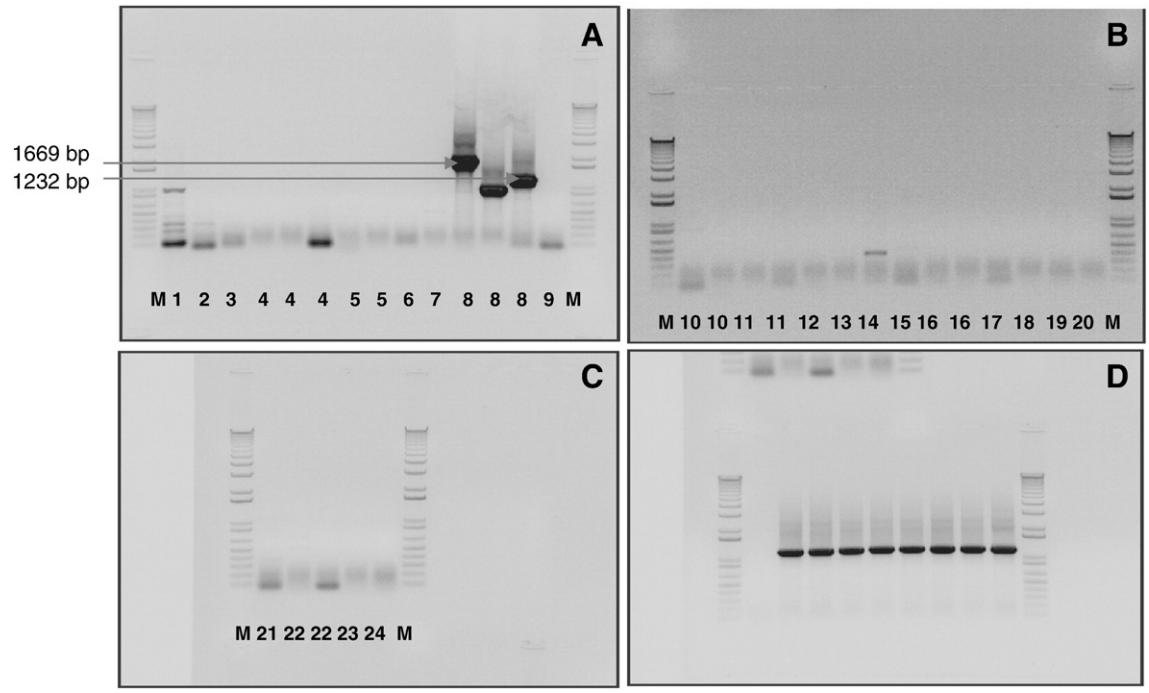

Fig. 1. Electrophoretic analysis of cDNA products from Seg-2 and Seg-7 of BTV-8 NET2006/04 isolate using the type specific primer pairs against 24 BTV types and generic primers for Seg-7. PCR amplicons were generated from cDNA of BTV-8 isolate NET2006/04 with three type 8 specific primer pairs (lane 8) (Mertens et al., 2007b) as indicated in panel A. No amplification was seen with primer pairs of other serotypes (Lanes 1 to 7 and 9-24-Panel A–C). Panel D showed the amplification of BTV RNA with Seg-7 specific primers (Anthony et al., 2007). Lane M: 1 kb marker.

also showed high levels of identity to BTV-2 from Corsica and BTV-8 from the Netherlands (91% identity), but only 78.7–80.1% identity to BTV-2 Taiwan (AY493686), indicating a clear separation into eastern and western groups (Fig. 1 — supplementary data).

Genome segment 2 encodes VP2, the outermost component of the virus capsid and the most variable of the BTV proteins (Maan et al., 2007a). Although Seg-2 can vary in length between different BTV types, it usually has a uniform length within each serotype, or Seg-2 nucleotype (Maan et al., 2007a). Seg-2 of BTV-8 (including NET2006/04) is conserved at 2939 bp, encoding a protein of 961 aa (Table 1). It has upstream and downstream NCRs of 17 bp and 36 bp, which show 100% and 97.2% identity respectively to Seg-2 NCRs of the other BTV-8 strains analyzed.

The full-length sequence of Seg-2 from NET2006/04 did not group closely with any of the other European strains analysed (Fig. 2A). It clustered closely with the reference strain of BTV-8 (93.4% nt identity), confirming its initial identification by RT-PCR. In contrast it showed only 45% to 71.1% nt identity to reference strains of the other 23 BTV serotypes. NET2006/04 showed some similarity to reference strains of BTV-18 (71.1%) and BTV-23 (69.8%), which have previously been grouped with BTV-8 within 'Seg-2, nucleotype D' (Maan et al., 2007a).

Seg-2 of the different BTV-8 isolates analysed showed 92.4–99.9% nt identity to the BTV-8 reference strain (RSArrrr/08). Seg-2 of the BTV-8 vaccine strain had only 9 nt differences from RSArrrr/08 (99.7% nt identity) from which it was derived (isolated in South Africa in 1937: reviewed by Alpar et al., in press). Although Seg-2 of BTV types 1, 2, 9 and 16 all showed evidence for separation into east–west topotypes, within each serotype (Fig. 2A), the BTV-4 and 8 strains analyzed were all from western origins; consequently there was no evidence for separation of Seg-2 into eastern and western groups within these two serotypes. NET2006/04 was most closely related (97% identity) to BTV-8 from Nigeria (NIG1982/07) but showed less similarity (93.4%) to the BTV-8 vaccine strain, indicating a more distant relationship. The recent South African isolates of BTV-8 (RSA1998/01, RSA1992/01 and RSA1987/01) are closely related to each other, with 97.8% to 99.6% nt identity (based on ~1000 bp region at the downstream end of Seg-2). However, they are more distantly related to NET2006/04 (94.1–94.7% nt identity) and the earlier South African strains (92.4–93.6% nt identity — Figs. 2A and B). Sequence data for Seg-2 of multiple northern European isolates of BTV-8 (2006–2008) showed that they are all closely grouped with NET2006/04 (>99.8% identity — including France (FRA2006/01) and the UK (UKG2007/05)).

Genome segment 3 encodes the highly conserved BTV sub-core-shell protein, VP3(T2). Seg-3 of the different European BTV strains is conserved at 2772 nt, encoding 901 aa, with upstream and downstream NCRs of 17 and 49 bases respectively (Table 1). Seg-3 of NET2006/04 showed >95% nt similarity to other 'western' group viruses, but only 80.9%–81.1% similarity to the 'eastern' European isolates analysed, which form a distinct cluster with >90.7% identity (Fig. 2 — supplementary data).

Genome Segment 4 encodes the highly conserved BTV capping and transmethylase enzyme — VP4(CaP). Seg-4 was 1981 nt long (regardless of BTV strain) encoding 644 aa, with a fully conserved 8 bp upstream NCR (Table 1). In contrast the 38 bp downstream NCR was more variable, showing 97.3% identity between the western strains, which show only 92% identity with BTV-2 from Taiwan (Table 1 — supplementary data). The full-length nt sequence of Seg-4 from NET2006/04 showed >78.6% nt identity to other BTV strains, grouping it with the other 'western' isolates (which showed 88.8–99.7% nt identity — Fig. 3— supplementary data). In contrast, BTV-2 from Taiwan (Ac. No. AY493689) showed only 78.6 to 80.1% to the 'western' viruses. The western group contained two subgroups, the larger of which included prototype strains of serotype 10, 11, 13 and 17 from the USA. NET2006/04 shows 80.1% nt identity to BTV-2 Taiwan, and is included in the 2nd subgroup (along with BTV-2 strains from the USA (Ac. No. L08637), Corsica (Ac. No. AY129085) and South Africa (vaccine strain — Ac. No. AY134477)), which shows a slightly closer relationship to BTV-2 Taiwan. Within this subgroup NET2006/04 is most closely related to the Corsican and South Africa (vaccine) strains (95.7% and 94.8% identity respectively).

Table 1
Characteristics of dsRNA genome segments (cDNA copy) and proteins of the bluetongue virus serotype 8 Netherland (NET2006/04)

†Genome segment (size: bp)	ORFs Bp (including stop codon)	Protein nomenclature (§: protein structure/ function)	Number of amino acids (Da)	Location	Accession numbers	5′ Terminal sequences of the positive strand	3′ Terminal sequences of the positive strand
1 (3944)	12–3920	VP1 (Pol)	1302 (149,834)	Within the sub-core at the 5 fold axis	AM498051	5′-GTTAAAATGCAATGGTCGCA	TGAGAGCACGCGCCGCATTACCCTTAC-3′
2 (2939)	18–2903	VP2	961 (111,317)	Outer capsid	AM498052	5′-GTTAAAATAGCGTCGCGATG	TAGCTCTCGTGACTGAGAGCTCGCGCGCTATCA ACTTAC-3′
3 (2772)	18–2723	VP3 (T2)	901 (103,265)	Sub-core capsid layer (T = 2 symmetry)	AM498053	5′-GTTAAATTTCCGTAGCCATG	TAGATGTGCGACCAATCTATGCACTTGGTAGCG GCAGCGGGAACACACTTAC-3′
4 (1981)	9–1943	VP4 (Cap)	644 (75,147)	Within the sub-core at the 5 fold axis	AM498054	5′-GTTAAAACATGCCTGAGCCA	TAATGCGTGACTGCTAGGTAAGGGGGGCCTTTA CAACTTAC-3′
5 (1776)	35–1693	NS1 (TuP)	552 (64,446)	Cytoplasm forms tubules	AM498055	5′-GTTAAAAAAGTTCTCTAGTTGGCAACCACCAAACATG	TAGTTACTGACTTCTGTTTTCTGTTTTTTCATTC TTCTTTCTACTTCTATTTTCTCTTAGCACTCTACT AGAACTTTTCAACTTAC-3′
6 (1637)	28–1608	VP5	526 (59,339)	Outer capsid	AM498056	5′-GTTAAAAAAGCGATCGCTCTCGCGAAGATG	TGAGCGCAGCGGAGCCACCGCTTTCCACTTAC 3′
7 (1156)	18–1067	VP7 (T13)	349 (38,558)	Outer core (T = 13 symmetry)	AM498057	5′-GTTAAAAATCTATAGAGATG	TAGTCCACTTTGCACGGGTGTGGGTTACATATG CGGTGTGTCGGTTGTGGGATATATGTAACCCAT TCAAACGTCTCTTAGATTACACTTAC-3′
8 (1125)	20–1084	NS2 (ViP)	354 (40,639)	Cytoplasm, viral inclusion bodies (VIB)	AM498058	5′-GTTAAAAAATCCTTGAGTCATG	TAGGCGCTTGTGACCGCGTGGTTGGGGGGGGA TTTTACACTTAC-3′
9 (1049)	16–1005	VP6 (Hel)	329 (35,568)	Within the sub-core at the 5 fold axis	AM498059	5′-GTTAAAAAATCGCATATGTCA	TAAAGGGTCCAGGGTACTCTCCTGACGTAGGGC GATTTACATCTTAC-3′
10 (822)	20–709	NS3	229 (25,514)	Cell membrane	AM498060	5′-GTTAAAAAGTGTCGCTGCCATG	TGAGGACAGTAGGTAGAGTGGCGCCCCGAGGT TTACGTCGTGCAGGGTGGTTGACCTCGCGGCGT AGACTCCCACTGCTGTATAACGGGGGAGGGTG CGCGACACTACACACTTAC-3′
	59–709	NS3a	216 (23,953)			5′ - GTTAAAAAGTGTCGCTGCCATGCTATCCGGGCTGATCCA AAGGTTCGAAGAAGAAAAAATG	TGAGGACAGTAGGTAGAGTGGCGCCCCGAGGT TTACGTCGTGCAGGGTGGTTGACCTCGCGGCGT AGACTCCCACTGCTGTATAACGGGGGAGGGTG CGCGACACTACACACTTAC-3′

Pol = RNA polymerase; Cap = capping enzyme (guanylyltransferase); Hel = helicase enzyme [42]; T2 = protein with T = 2 symmetry; T13 = Protein with T = 13 symmetry; ViP = viral inclusion body matrix protein; TuP = tubule protein. Letters in bold blue are the 5′ and 3′ terminal conserved sequences in genome segments of BTV-8 NET2006/04. Letters in red are the substitution of C for A at sixth position from 3′ end in genome segment 1 in NET2006/06, and T for A in genome segment 9, in NET2006/06 and six other western viruses (out of 41 strains analysed).

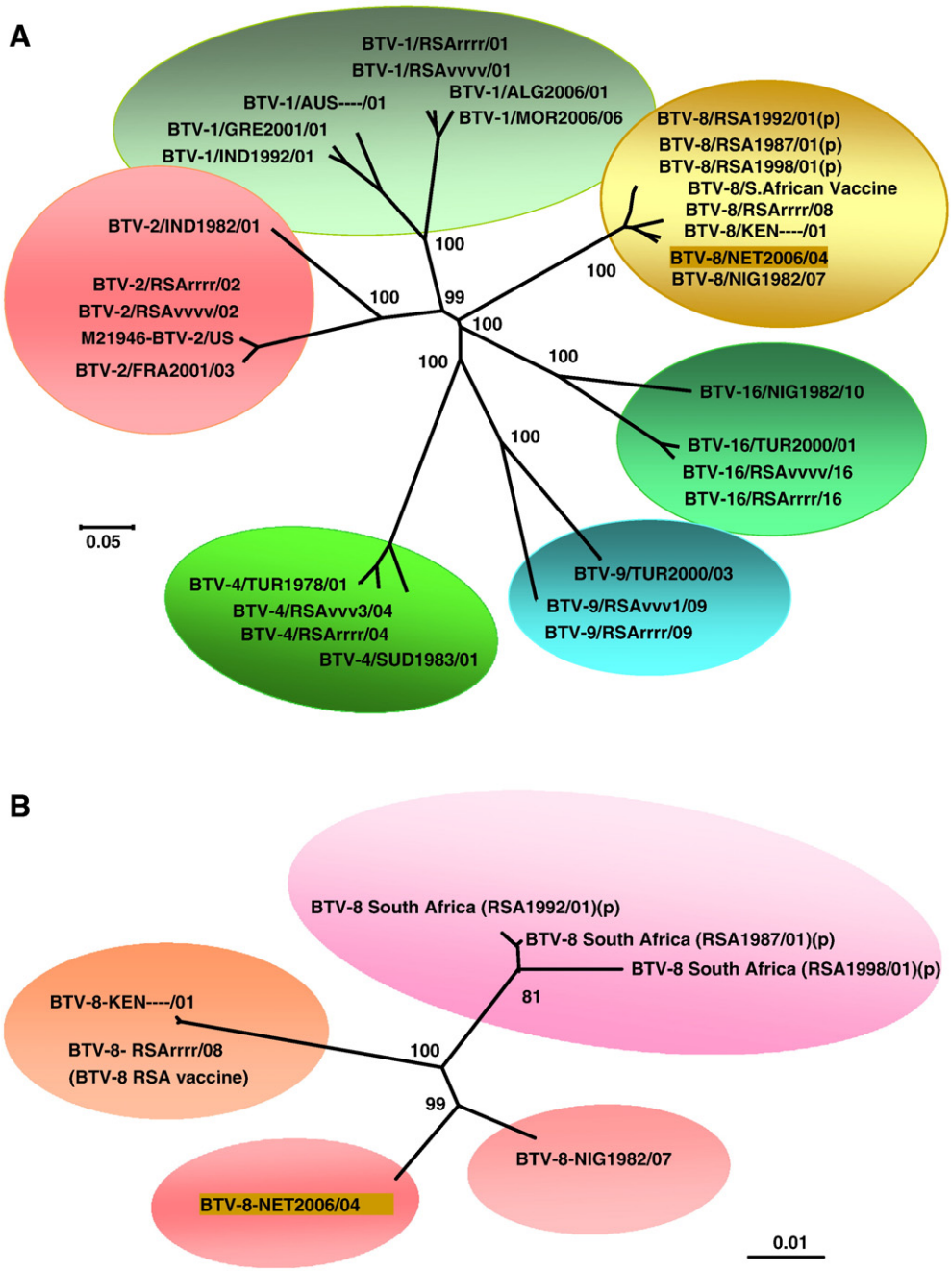

Fig. 2. A: Neighbour-joining tree showing the relationships between nucleotide sequences of Seg-2 from European strains of BTV serotype 1, 2, 4, 8, 9 and 16. The tree was constructed using distance matrices, generated using the p-distance determination algorithm in MEGA 3 (2000 bootstrap replicates) (Kumar et al., 2004). The numbers indicate bootstrap confidence values after 2000 replications. p indicates partial sequences. The trees shown in Figs. 2B–6 were drawn using same parameters. B: Neighbour-joining tree showing relationships between nucleotide sequences of Seg-2 from strains of BTV-8.

Genome segment 5 of NET2006/04 was compared with European field strains, and the South African BTV-vaccine strains (Table 1 and Fig. 4 — supplementary data). Seg-5 of NET2006/04 is 1776 nt long, encoding NS1(TuP) at 552 aa, with upstream and downstream NCRs of 34 and 83 bp in length (Table 1). Seg-5 from the different BTV isolates formed two distinct clusters, representing eastern and western lineages with 82.2–83.5% identity between members of these different groups. The eastern group included European field strains of BTV-1 (from Greece), 9 and 16, as well as the vaccine strain of BTV-16. However, NET2006/04 clusters with the 'western' viruses, including European field strains of BTV-2 and 4, and the South African vaccine strains of types 1, 2, 4 and 9.

Seg-5 of NET2006/04 was most closely related to BTV-4 from Greece (GRE1999/15 — 97.5% identity) providing the first sequence data indicating that the northern European outbreak in 2006 was caused by a 'western' virus. Within the eastern group, the Sardinian field isolates of BTV-16 (SAD2004/03 and SAD2004/04) were almost identical to the BTV-16 South African vaccine strain, with 99.9% nt similarity, indicating a very recent common ancestry. Within the western group, European field strains of BTV-2 from Spain (SPA2005/01) and BTV-16 from Italy (ITL2002 — Ac. No. DQ017960) were almost identical (99.9–100%) to Seg-5 of the South African BTV-2 vaccine strain (RSAvvvv/02-Ac. No. AM773684 and AY138895), indicating that a reassortment event has taken place between vaccine strains from different serotypes.

Genome segment 6 encodes VP5 — the smaller outer capsid component and second most variable of the BTV proteins. Seg-6 of the different BTV-8 isolates is 1637 bp long showing >94.7% nt identity, and encoding 526 aa (Table 1). The upstream and downstream NCRs are 27 and 29 bp long, with 96.2% and 100% identity respectively. Comparisons of Seg-6 from the European strains of BTV-1, 2, 4, 8, 9 and 16, identified seven main lineages, which primarily correlate with virus serotype (Fig. 3). However, the BTV-1 strains from the Mediterranean region included two distinct lineages, represented by an eastern strain (GRE2001/01) and a western strain (ALG2006/01 and MOR2006/06) (Fig. 3), reflecting two distinct Seg-6 topotypes and introductions of BTV-1 from different geographic origins. The Seg-6 sequence of GRE2001/01 showed a relatively large separation (79% similarity) from the BTV-1 vaccine strain (RSAvvvv/01), reflecting their derivation from eastern and western groups of viruses respectively. In contrast ALG2006/01 and MOR2006/06 showed 95% nt similarity to the vaccine strain, reflecting a closer geographical origin.

Seg-6 from different BTV-9 isolates also formed two distinct groups, containing 'eastern' strains from Europe 2000 to 2002, and the 'western' vaccine strain, respectively. However, these two groups are much more widely separated (30.4% variation) in a manner similar to Seg-6 from different BTV serotypes (Fig. 3). This not only indicates that Seg-2/VP2 sequences (and not Seg-6/VP5) determine the identity of BTV-9, but also that one or other of these lineages has acquired segment 6 by reassortment with a distinct virus type.

Seg-6 of NET2006/04 grouped with other BTV-8 isolates from Nigeria, Kenya and S. Africa (reference strain) with >94.8% similarity overall. As already noted for Seg-2 (encoding VP2), the closest strain to NET2006/04 was BTV-8 from Nigeria isolated during 1982 (NIG1982/07), with 97.7% nt similarity.

Genome segment 7 of NET2006/04 is 1156 bp long, encoding the 349 aa of the major BTV serogroup-specific antigen and core surface protein — VP7. The upstream and downstream NCRs are 17 and 89 bases long respectively (Table 1). The aa sequence of VP7 is significantly more conserved (>80.2% identity) than suggested by the nt sequence of Seg-7 of NET2006/04 which shows 75.5%–97% nt identity to the other BTV strains. This reflects large numbers of synonymous mutations in the third base position.

Unlike majority of the BTV genome segments, Seg-7 formed six distinct clusters. Three of these are primarily from a western origin (with the single exception of the Chinese strain of BTV-12) and three from an eastern origin (Fig. 4). Two other Mediterranean BTV strains (BTV-2 Tunisia — Ac. No. AF469115 and BTV-2 Corsica — Ac. No. AY079124), belonging to western group 2) were included in these comparisons, showing 79.3% and 80.1% identity with NET2006/04 respectively. Within western group 1, NET2006/04 clusters closely (97% identity) with BTV-1 Honduras (Ac. No. AF188670) and the South African BTV-1 reference strain (Ac. No. AF188669) (Fig. 4).

Genome segment 8 of BTV is conserved at 1125 bp, encoding the 354 aa of the viral inclusion body (VIB) matrix protein — NS2. The upstream and downstream NCRs (19 and 41 bp respectively) are fully conserved in the viruses compared (Table 1). Seg-8 of the European BTV isolates showed clear separation into eastern and western groups, with NET2006/04, vaccine strains of BTV-2, 4 and 9 and European field strains of BTV-2 and 4, in the western group (Fig. 5). Overall nt identities of 76.1% to 80.4% were detected between the eastern and western groups. Preliminary sequence data for Seg-8 of BTV-1 from Greece (GRE2001/01) and BTV-16 from Turkey (TUR2000/01) indicate that they both group within the eastern cluster. Seg-8 of NET2006/04 showed 99.8% similarity to a distinct isolate from the same outbreak in the Netherlands, but from a different animal (NET2006/01). It also showed relatively high levels of nt similarity to the South African vaccine strains of BTV-2 (Ac. No. AY138896 — 96.6%), BTV-4 (Ac. No. AY857502 — 95.5%) and BTV-9 (RSArrr1/09 -Ac. No. AM900374 — 95.6%), as well as to the Corsican field strains of BTV-4 (Ac. No. AY857499 — 96%) and BTV-2 (Ac. No. AY124372 — 92.8%).

Genome segment 9 codes for minor core protein VP6, the core associated helicase of BTV. The Seg-9 nt sequences were divided into eastern and western groups (Fig. 5— supplementary data). Seg-9 of the eastern BTV isolates is 1052 bp, encoding a protein 330 aa in length, while Seg-9 from the western lineage is 1049 bp (329 aa) (Table 1). The upstream NCR is 14–15 bp (W–E), while the downstream NCR is 44 bp. Seg-9 of NET2006/04 belongs to the western group and is closely related to the Corsican isolate of BTV-2 (Ac. No. AY124373 — 97.1% identity) and the South African BTV-2 vaccine strain (Ac. No. AF530066 — 94.2% identity), but is more distantly

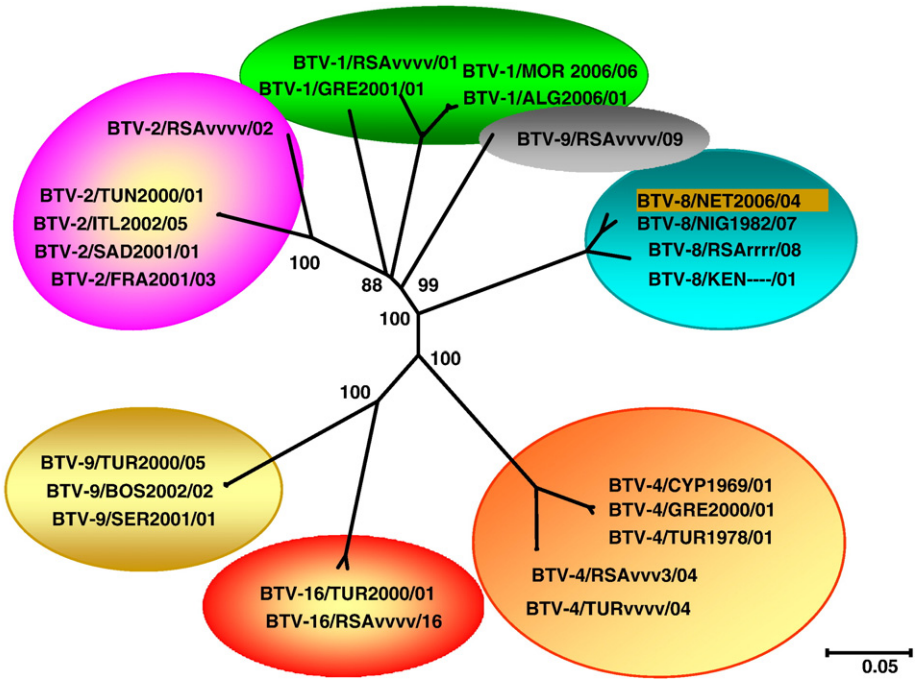

Fig. 3. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-6 from European strains of BTV serotype 1, 2, 4, 8, 9 and 16.

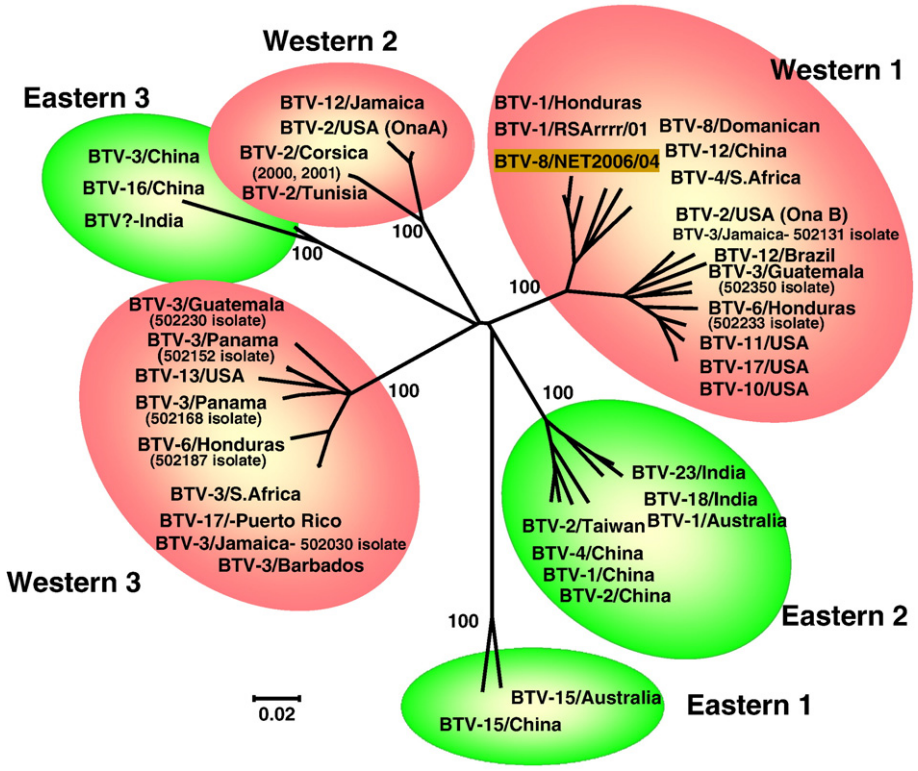

Fig. 4. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-7 from different BTV serotypes.

related to the eastern strains of BTV-1-GRE2001/01 (76.3% identity), BTV-9-BOS2002/02 and BTV-16-TUR2000/01 (76.6% identity). Overall Seg-9 showed >75.8% nt similarity across the eastern and western strains of BTV.

Genome segment 10 of NET2006/04 is 822 bp long, coding for the small non-structural proteins NS3 (229 aa) and NS3a (216 aa) (Table 1).

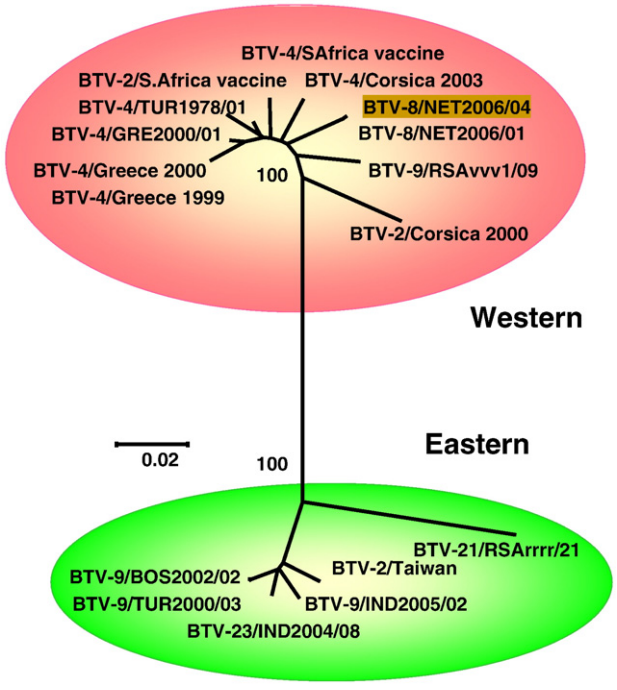

Fig. 5. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-8 from BTV isolates from Europe and around the world.

The upstream and downstream NCRs are 19 and 113 bp long respectively. Seg-10 which is >82% conserved, across all of the isolates compared, can be divided into four groups, two from eastern and two from western origins. Seg-10 from European isolates of BTV-1, 9 and 16 cluster together in eastern group 1 (equivalent to clade A1 of Seg-10 — reported by Balasuriya et al., 2008), while the European isolates of BTV-2 and 4, and South African reference strain of type 1 cluster within western group 2 (equivalent to sub-clade A3 within clade A2 — Balasuriya et al., 2008) (Fig. 6). Seg-10 of the South African vaccine strains of BTV-1, 2, 3, and 4, cluster within western group 1 (equivalent to clade B2 — Balasuriya et al., 2008). NET2006/04, is included in this group and was most closely related to the vaccine strains of BTV-2 (Ac. No. AF481094— 96.2% identity), BTV-4 (Ac. No. AF512908 — 93.8% identity), BTV-3 (Ac. No. AF512918 — 92.3% identity), and BTV-1 (Ac. No. AF512910 — 92.2% identity). But was more distantly related (<82.9% identity) to the European BTV isolates in eastern group 1 or western group 2.

Conserved terminal sequences and stop codons

The upstream and downstream terminal hexanucleotides (Mertens et al., 2007e) are maintained for the majority of the BTV genome segments included in these analyses (Table 1). However, a substitution of C to A was detected at the sixth position from the 3′ end of Seg-1 from NET2006/04. Another substitution of T for A was detected at the sixth position from the downstream end of Seg-9 from NET2006/06 and six of the other western viruses (out of 41 strains analysed).

The stop codon of Seg-1 is …UGA…, while Seg-4 and 9 use UAA, and Seg- 3, 5, 7 and 8 use UAG. The stop codon of Seg-6 is conserved as UGA across isolates of all 24 BTV serotypes. In contrast stop codons are not conserved in Seg-2 between different BTV types, and include … UGA…, …UAG… and …UAA… (Maan et al., 2007a), although all of the BTV-8 strains use …UAG….. Seg-10 of NET2006/04, BTV-1 and BTV-2 vaccine strains, (all of which are in western group 2) use …UGA… The other field strains and the BTV-8 vaccine strain (that are included in

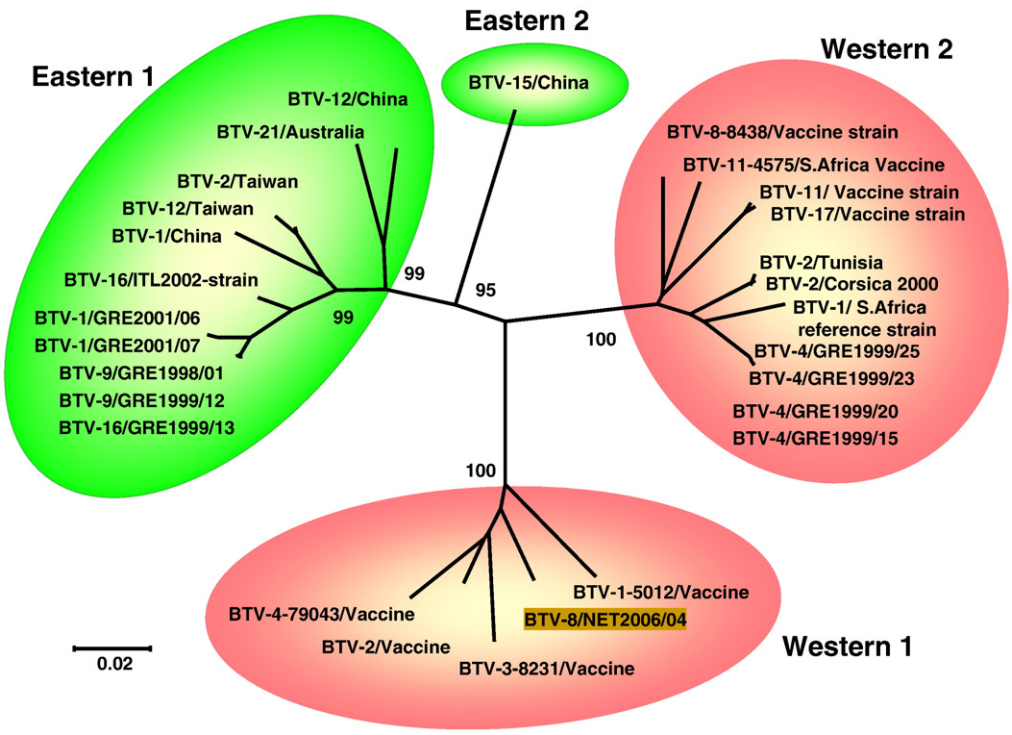

Fig. 6. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-10 from BTV isolates from Europe and around the world.

western group 1), together with both of the eastern subgroups, all use …UAA….

Discussion

In August 2006, sheep in the Maastricht region of the Netherlands started showing severe clinical signs that were typical of 'bluetongue' disease. Blood samples were sent to the Central Institute for Animal Disease Control (CIDC) at Lelystad in the Netherlands, where BTV-specific antibodies were detected by ELISA, and the presence of BTV was confirmed by RT-PCR targeting Seg-10 (Toussaint et al., 2007; Vandenbussche et al., in press). This was the first time that an outbreak of bluetongue had ever been identified in northern Europe. Further blood samples were sent to the CRL at IAH Pirbright in the UK, where competition ELISA confirmed that these samples were positive for BTV-specific antibodies (Batten et al., 2008; Vandenbussche et al., in press). A *C. sonorensis* cell line (KC cells) and BHK cells were used to isolate virus (IAH reference collection isolate number NET2006/04), which was subsequently identified as BTV by conventional and real-time RT-PCR assays targeting Seg-7 and Seg-1 respectively (Anthony et al., 2007; Shaw et al., 2007).

We report the first identification of the northern European strain (NET2006/04) as BTV-8, by conventional RT-PCR assays using serotype-specific primers targeting Seg-2 (Mertens et al., 2007f, g), together with a complete nucleotide sequence for the genome of NET2006/04. Phylogenetic analyses showed that all of the genome segments of this isolate contain significant numbers of nucleotide changes, compared to segments (where published) of the BTV vaccine or field strains that were used, or already circulating in southern Europe. This supports serological evidence that NET2006/04 represents the novel introduction of an exotic BTV strain/serotype into Europe, although details of its entry route to the region remain unknown. These analyses indicate that NET2006/04 is not derived from the BTV-8 vaccine strain, which was used as part of the South African 'Group B' multivalent live attenuated vaccine (containing BTV-3, 8, 9, 10 and 11) in Bulgaria during 2000 (Panagiotatos, 2004; Savini et al., in press). However, there was some serological evidence indicating BTV-8 infections of sentinel animals in Bulgaria during 2006, even though attempts to isolate or detect the virus by RT-PCR were unsuccessful (OIE, 2006b).

The situation that has occurred in Europe since 1998 is unique, with the introduction of multiple field strains from western (BTV-1, 2, 4, 8) and eastern (BTV-1, 9, 16) lineages. Live attenuated monovalent 'vaccine' strains of BTV-2, 4, 8, 9 (western group) and BTV-16 (eastern group) have also been used in the Mediterranean region, in attempts to minimise virus circulation. The release of these vaccine strains, some of which (including BTV-2 and 16) have persisted in the field, has added further genetic diversity generating an unprecedented mix of field and vaccine strain viruses, from both eastern and western lineages within southern Europe. This has provided multiple opportunities for the exchange/reassortment of genome segments between different strains, as already reported between strains of BTV-2 and 16 (Batten et al., in press; Monaco et al., 2005).

Genome segments from European and (selected) other BTV strains from around the world were compared. Variations were detected in each of the segments that correlate with their geographic origins, dividing them into eastern and western topotypes/groups (Fig. 6 — Supplementary data). All of the genome segments of NET2006/04 grouped with other 'western' viruses. The east/west grouping appears to be a dominant characteristic of the *Orbivirus* phylogeny, supporting observations previously made for Seg-3 of BTV and EHDV (Cheney et al., 1995; Gould and Pritchard 1991; Pritchard et al., 1995).

The BTV outer capsid proteins VP2 and to a lesser extent VP5 (encoded by genome segments 2 and 6) interact with neutralising antibodies and are the most variable of the BTV proteins/genome segments (Fig. 6 — Supplementary data) (Cowley and Gorman 1989; Huismans et al., 1987; Maan et al., 2007a; Singh et al., 2004). Sequencing studies and phylogenetic comparisons of Seg-2 from different BTV isolates can be used to identify the 24 BTV serotypes (Maan et al., 2007a) and provide a basis for serotype-specific RT-PCR assays (Mertens et al., 2007f, 2007g). These methods were used successfully to identify different BTV types

that have invaded Europe and the USA over the last 10 years (Johnson et al., 2000, 2007; Mertens et al., 2007f,g; Potgieter et al., 2005; Zientara et al., 2006). In the studies described here, only Seg-2 and Seg-6 showed variations that correlate with virus serotype, confirming the initial identification (by RT-PCR) of NET2006/04 as BTV-8. BTV-8 has previously been isolated from Kenya, Nigeria, South Africa, South and Central America (Mo et al., 1994), and India (Daniels et al., 2004; Prasad et al., in press). Seg-2 of NET2006/04 was most closely related to BTV-8 from Nigeria in 1982 (NIG1982/07), indicating that the northern European strain originated in sub-Saharan Africa. It also showed a close relationship in the more conserved genome segments with the Corsican strains of BTV-2 or BTV-4 (2000 onwards), which were also recently derived from an African lineage.

Seg-3 showed up to 99.8% nt similarity between the BTV-2 strains from Corsica and mainland Italy, with BTV-4 strains from Morocco and Spain indicating that (despite belonging to different serotypes) these strains all share Seg-3 from a recent common ancestor, providing evidence of genome segment exchange/reassortment. Despite also belonging to different serotypes, Seg-5 from the 1999 strain of BTV-16 from Greece (GRE1999/13) is also very similar to BTV-9 from Bulgaria (99.9%) indicating that they have also been involved in a recent reassortment event.

The clear separation of the majority of the BTV genome segments into eastern and western groups indicates that the viruses in these different regions have been separated and have acquired point mutations over a relatively long period of time, with little or no mixing or exchange between them. The existence of distinct eastern and western topotypes of Seg-2 and Seg-6, within individual BTV serotypes, also suggests that these different 'types' diverged and became genetically distinct as an initial step. Individual strains or lineages of each type subsequently became separated (geographically), allowing them to accumulate point mutations, and generating distinct topotypes within the each serotype.

Genome Seg-7 and Seg-10 also showed major variations that do not simply reflect the geographic origins of the virus isolate (Fig. 6 — Supplementary data). Seg-10 is the fourth most variable segment of the virus strains compared here, which separated into 4 distinct groups, two eastern and two western (although one of the eastern groups is represented by only a single strain of BTV-15 from China — Nikolakaki et al., 2005). The significance of these variations in Seg-10 remains uncertain, however they may relate to the role of NS3/NS3a in the release of progeny virus particles from the host cell (Hyatt et al., 1989). In a recent extensive study of sequence variations in BTV Seg-10, Balasuriya et al. (2008) identified two main groups (A and B) with a number of sub-clades. Some of these groups (clades A4, B1 and B3) containing viruses from the Americas, and the BTV-9 vaccine strain, were not included in the study presented here. However, sub-clades A1, A2–A3 and B2 appear to be equivalent to eastern group1, western group 2 and western group 1 respectively. The Chinese isolate of BTV-15, which forms the eastern group 2 identified here, was not included in the study by Balasuriya et al. (2008), although it appears likely that it would form a further distinct clade or sub-clade.

Wilson et al. (2000) compared BTV Seg-7 sequences, primarily from western isolates, showing several distinct clades that appear to form three distinct groups. These authors also identified two eastern strains that did not fit within these western groups. The sequences compared here indicate that Seg-7 is the third most variable of the BTV genome segments (Fig. 6 — Supplementary data). Seg-7 separated into six distinct groups, three of which fit with the groups described by Wilson et al. (2000), containing mainly western viruses (with the exception of BTV-12 from China, which grouped along with NET2006/04 within western group 1). The three other groups all contained eastern viruses. However, unlike Seg-7, Seg-10 of BTV-12 from China groups with other eastern strains, suggesting that this virus acquired a 'western' Seg-7 by reassortment, possibly from an exotic strain of the virus introduced into the region.

The data presented here provide the first complete sequence (segments 1–10) of the northern European strain of BTV-8. Although subsequent data for other isolates from the European outbreak in 2006 to 2008 have detected small numbers of nucleotide changes in several genome segments, they confirm that the strains were all closely related, representing a single virus lineage. Comparisons of NET2006/04 with the sequences of subsequent isolates will be used to track virus movements at different stages (years) of the outbreak. These comparisons will also help to identify any subsequent reassortment events involving this virus strain, and help to identify other related strains from different locations.

Materials and methods

Virus isolates, virus isolation and propagation in cell culture

Virus isolates from the reference collection at IAH Pirbright are referred to by their reference collection number, with the generic format 'three letter country code' year of isolation/isolate number for that year. Table 1 (supplementary data) gives details concerning the country of origin, year of isolation and EMBL/GenBank accession numbers for the isolates used in this study.

Blood samples (containing EDTA) taken from pyrexic animals at the start of the 2006 Netherlands outbreak of BT, were provided by Dr. Eugène van Rooij — CIDC-Lelystad, Netherlands. A 3.0 ml aliquot of each EDTA blood sample was washed three times with 10 ml of sterile phosphate-buffered saline (PBS). The red blood cells (RBC) were centrifuged at 3000 ×*g* for 5 min at 4 °C and the supernatant discarded. After the final wash, the RBCs were resuspended in 3.0 ml of PBS. RNA was extracted from 1.0 ml of the washed RBC for RT-PCR. 1.0 ml of the washed RBC was injected into embryonated chicken eggs. The harvested material was designated BTV8-E1. The remainder of the washed RBC were used for virus isolation in *C. sonorensis* (KC) cells (originally provided by colleagues at the USDA lab in Laramie, Wyoming). At 7 dpi KC culture supernatants (inoculated with sample number A83/06-6, from 'sheep number 6') was used to infect BHK-21 cells. The resulting virus isolate (NET2006/04–KC1/BHK1) was used for sequencing studies. Further details concerning the origins and passage history of individual virus isolates can be found at Mertens et al. (2007d).

Isolation and purification of nucleic acids

RNA was extracted from cell-free supernatants, or EDTA treated blood samples, using the QIAamp Viral RNA Mini Kit (QIAGEN) as per manufacturer's protocol, The RNA required for synthesis of full-length cDNA copies of genome segments, for sequencing (Attoui et al., 2000; Maan et al., 2007a,b), was purified from infected monolayers using Trizol® (Invitrogen), in accordance with manufacturer's instructions.

Identification and typing of the Netherland isolate (NET2006/04)

RNA from cell-free supernatants or EDTA treated blood samples, were used in RT-PCR assays. Serogroup-specific real-time RT-PCR assays targeting Seg-1 were carried out as described by Shaw et al. (2007). Conventional serogroup-specific RT-PCR assays targeting Seg-7 (Anthony et al., 2007), and serotype-specific assays (targeting Seg-2: Mertens et al., 2007f,g), were performed using a single tube RT-PCR method following the "One-Step RT-PCR kit" protocol (Qiagen, Courtaboeuf, France). Five microlitres of each RT-PCR was analyzed by electrophoresis on a 1% agarose gel.

Full length cDNA synthesis and amplification by PCR

Purified viral dsRNA was used for cDNA synthesis as described by Maan et al. (2007a,b). Briefly, ~400 ng of dsRNAs were ligated to a single-stranded anchor-primer using T4 RNA ligase (NEB) at 4 °C for

12 h. The oligo-ligated dsRNAs of BTV-8 isolate (NET2006/04) were converted to full length cDNAs using the AMV reverse transcription system (Promega) at 37 °C for 1 h. The DNA segments were then purified by agarose gel electrophoresis (AGE) and recovered from the gel as four pools: large genome segments (Seg-1, tube 1; Seg-2 and Seg-3, tube 2); medium (Seg-4 to 6, tube 3) and small (Seg-7 to10, tube 4). PCRs were performed on the cDNAs using the 'Triple Master PCR system' (Eppendorf) with a single primer complementary to the anchor-primer: denaturing at 95 °C for 30 s, then annealing and extension at 68 °C for 3 min, for 30 cycles.

Sequence determination of genome segments

Full length cDNA amplicons were generated (in four separate reactions: tubes 1 to 4) for all 10 genome segments of NET2006/04. Each cDNA was purified by 1% TAE agarose gel electrophoresis (AGE), excised from the gel and purified using a GFX™ PCR DNA and gel band purification kit' (Amersham Pharmacia Biotech, Inc) as per the manufacturer's instructions. The purified cDNA of each amplicon was used for direct sequencing, in both directions, using BTV-specific 'phased-primers' (Maan et al., 2007b) and 'Cycle Sequencing Ready Reaction' kit (CEQ DTCS Beckman Coulter) on a Beckman Capillary Sequencer. Internal forward and reverse primers were designed from the resulting terminal sequence data, generating full-length contiguous sequences of all 10 genome segments.

Sequence analysis

Consensus sequences from each segment were assembled and analyzed using SeqMan Software (DNAStar Inc.). All sequence alignments were performed with the CLUSTAL W software (Thompson et al., 1994). Phylogenetic trees were constructed by neighbour-joining using distance matrices generated by the p-distance determination algorithm in MEGA 3 (2000 bootstrap replicates) (Kumar et al., 2004). Sequence relatedness is reported as percentage identity. The sequences obtained for each genome segment has been submitted to EMBL and the accession numbers obtained are AM498051 to AM498060 (Table 1). For accession numbers of previously published sequences see: Mertens et al. (2007a) and Table 1 — supplementary data.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.04.028.

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Appendix 7

Analysis of data for Chapter 5

Data on viral replication (either $\log_{10}$ $TCID_{50}$ or Ct values over time when incubated at different temperatures) were modelled using logistic growth curves with a common temperature-dependent growth rate. Specifically, the virus level ($Y_j(t,T)$) for strain j at time t when incubated at temperature T is given by,

$$Y_j(t,T) = \begin{cases} Y_0 & T \le T_{\min}^{(j)}, \\ \dfrac{\kappa_j}{1+\left(\dfrac{\kappa_j - Y_0}{Y_0}\right)\exp\ -\beta_j(T)t)} & T > T_{\min}^{(j)}, \end{cases} \quad (1)$$

where Y_0 and κ are the initial and final virus levels, respectively, and,

$$\beta_j(T) = \begin{cases} 0 & T \le T_{\min}^{(j)}, \\ \alpha_j(T - T_{\min}^{(j)}) & T > T_{\min}^{(j)}, \end{cases} \quad (2)$$

is the temperature-dependent replication rate ($T_{\min}$ is the threshold temperature for viral replication and α is the replication rate above the threshold).

Differences between virus strains were incorporated by assuming hierarchical structure for the model parameters, so that the parameters for strain j are drawn from higher-order distributions, namely,

$$\begin{aligned} \kappa_j &\sim \mathrm{N}(\mu_\kappa, \sigma_\kappa^2), \\ T_{\min}^{(j)} &\sim \mathrm{N}(\mu_{T_{\min}}, \sigma_{T_{\min}}^2), \\ \alpha_j &\sim \mathrm{Gamma}(a_\alpha, b_\alpha), \end{aligned}$$

while the initial virus levels for each curve were modelled as,

$$Y_0^{(curve)} \sim \mathrm{N}(\mu_{Y_0}, \sigma_{Y_0}^2),$$

where the μs, σs, as and bs are higher-order parameters.

Model parameters were estimated in a Bayesian framework. The likelihood for the virus titre data is,

$$L_V(\mathbf{V} \mid \boldsymbol{\theta}) = \prod_j \prod_r \prod_t \prod_T f(V_{jrtT} \mid Y_j(t,T), \sigma_V^2), \quad (3)$$

where V_{jrtT} is the observed titre for strain j in replicate r at time t when incubated at temperature T, f is the probability density function for the Normal distribution, $Y_j(t,T)$ is the

expected titre (given by equation (1)) and σ_V^2 is the error variance for the virus titre data. The corresponding likelihood for the Ct value data is,

$$L_C(\mathbf{C} \mid \boldsymbol{\theta}) = \prod_j \prod_r \prod_t \prod_T f(C_{jrtT} \mid Y_j(t,T), \sigma_C^2), \quad (4)$$

where C_{jrtT} is the observed Ct value for strain j in replicate r at time t when incubated at temperature T, f is the probability density function for the Normal distribution, $Y_j(t,T)$ is the expected Ct value (given by equation (1)) and σ_C^2 is the error variance for the Ct data.

Priors for the strain-specific parameters were given by the higher-order distributions, while non-informative (diffuse Normal or diffuse gamma) priors were assumed for the higher-order parameters and the error variances.

The methods were implemented in OpenBUGS (http://www.openbugs.info). For each data-set, two chains each of 300,000 iterations were generated (with the first 100,000 iterations discarded to allow for burn-in of the chains). Chains were subsequently thinned (by selecting every fiftieth iteration) to reduce autocorrelation amongst the samples. Convergence of the chains was monitored using Gelman-Rubin statistics.

Appendix 8

Spearmen-Karber formula (Finney, 1964)

Log_{10} Median dose = X_0- d/2 + d* (Σ r/n)
where:
X_0 is the reciprocal of the Log_{10} value of the lowest dilution where all test wells are positive
d is the Log_{10} dilution factor
n is the number of wells/ dilution
r is the number of positive wells for each dilution
Σ r/n is the sum of the proportion of positive wells, beginning at the lowest dilution showing 100% positive results
The obtained value would be log_{10} $TCID_{50}$/100µl so add 1 to get the log_{10} TCID50/ml

Example:

0	+	+	+	+
-1	+	+	+	+
-2	+	+	-	-
-3	+	-	-	-
-4	-	-	-	-

X_0 – the lowest dilution where all wells are positive is -1, the reciprocal of this is **1**

d – the dilution is a ten-fold one, the log_{10} of this is **1**

Σ r/n - -1 dilution is 4/4 = 1
-2 dilution is 2/4 = 0.5
-3 dilution is 1/4 = 0.25
Total = 1.75

Log_{10}Median Dose = X_0 – d/2 + d*(Σ r/n)

= 1 – 1/2 + 1*(1.75)
= 1 – 0.5 + 1.75
= 2.25

This is per inoculum (100 µl usually). To get this converted to 1 ml, add 1.

Result: Log_{10} TCID50/ml : 3.35
or $10^{3.25}$ TCID50/ml
or 1.7 x 10^3 TCID50/ml