MOLECULAR DETERMINANTS OF BLUETONGUE VIRUS VIRULENCE

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Abstract

Bluetongue virus (BTV) is an arbovirus and the cause of “bluetongue”, a major infectious disease of ruminants. Whilst the BTV structure and replication strategies are well elucidated, less is known on the genetic variability of BTV and the molecular determinants affecting virus-host interactions.

In order to investigate the determinants of BTV virulence, in this study, we compared the phenotype and genotype of a highly virulent strain of BTV-8 isolated in the Netherlands a passaged minimally in tissue culture ($BTV8_L$), with a strain passaged extensively in tissue culture ($BTV8_H$). $BTV8_L$ was shown to be highly pathogenic in sheep and in a mouse model of bluetongue, while $BTV8_H$ was attenuated in both hosts. Full genome sequencing revealed differences in 16 amino acid residues between these two strains. Using reverse genetics, we rescued both viruses, in order two further dissect their biological features. Rescued viruses retained the phenotype of the parental viruses *in vivo* and *in vitro*. Reassortants between $BTV8_L$ and $BTV8_H$ showed that mutations in several segments contributed to attenuation of the high passage virus. The major determinants of BTV8 virulence in $IFNAR^{-/-}$ mice were shown to be located in segments 1, 2, 6 and 10. *In vitro* studies of selected reassortants showed that through extensive passage in tissue culture $BTV8_H$ acquired increased affinity for glycosaminoglycans. This property was conferred by mutations in segment 2 and resulted in increased yields of the virus *in vitro* and attenuation *in vivo*. Additionally, $BTV8_H$ was unable to replicate in IFN competent primary sheep endothelial cells. Our data showed that multiple segments were involved in decreased efficiency of $BTV8_H$ replication in cells in an IFN-induced antiviral state. Moreover, we examined changes in viral population diversity that occured after BTV-8 isolation in insect cells (*Culicoides*, KC) and after passage in mammalian cells and linked decreased diversity with BTV virulence *in vivo*. We found, that in general, the number of genetic variants was higher in BTV-8 before cell passaging, or after one passage in KC cells, compared to the number observed after even a single passage in BHK-21 cells. The highest total number of variants was found in virus passaged in KC cells, which suggests that insect vector might serve as an amplifier of quasispecies diversity of BTV.

Together, these findings suggest that the virulence of BTV is a multifactorial phenomenon involving many aspects of virus-host interactions and it is not only affected by changes in the viral proteins selected at the consensus level, but also by the genetic variability of the population as a whole.

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List of accompanying material

The following publications, resulting from the work carried out in this project, are attached to this thesis.

Janowicz, A., Caporale, M., Shaw, A., Gulletta, S., Di Gialleonardo, L., Ratinier, M., & Palmarini, M. (2015). Multiple Genome Segments Determine Virulence of Bluetongue Virus Serotype 8. *Journal of Virology*, *89*(10), 5238–5249. doi:10.1128/JVI.00395-15

Caporale, M., Di Gialleonorado, L., Janowicz, A., Wilkie, G., Shaw, A., Savini, G., Van Rijn, P.A., Mertens, P., Di Ventura, M, Palmarini, M. (2014). Virus and Host Factors Affecting the Clinical Outcome of Bluetongue Virus Infection. *Journal of Virology*, *88*(18), 10399–10411. doi:10.1128/JVI.01641-14

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Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Anna Janowicz

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Abbreviations

2’OMTase	2' -O-methyltransferase
AHSV	African horse sickness virus
Actβ	Beta-actin
ATPase	Adenosine triphosphate synthase
BoEC	Bovine endothelial cells
bp	Base pairs
BTV	Bluetongue virus
CLP	Core like particles
CMV	Cytomegalovirus
COX-2	Cyclooxigenase-2
CPE	Cytopathic effect
DC	Dendritic cell
EEV	Equine encephalosis virus
EHDV	Epizootic haemorrhagic disease virus
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EMCV	Encephalomyocarditis virus
ESCRT	Endosomal sorting complexes required for transport
FACS	Fluorescence-activated cell sorting
FMDV	Foot and mouth disease virus
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
IFIT	Interferon induced proteins with tetratricopeptide repeats
IFN	Interferon
IFNAR	Interferon alpha/beta receptor
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
iRNA	RNA interference
ISG	Interferon stimulated genes
JAK	Janus kinase
Mda-5	Melanoma differentiation-associated protein 5
MLV	Modified live vaccine
MOI	Multiplicity of infection
MX	Myxovirus resistance gene

n	number
N7MTase	Guanine-N7-methyltransferase
nt	Nucleotide
NTP	Nucleotide triphosphate
OIE	World Organisation for Animal Health
ORF	Open reading frame
OvEC	Ovine endothelial cells
p.i.	Post infection
PCR	Polymerase chain reaction
PFU	Plaque forming units
qPCR	Quantitative polymerase chain reaction
RdRp	RNA depended RNA polymerase
RG	Reverse genetics
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RSA	Republic of South Africa
RSAD2	Radical S-adenosyl methionine domain containing 2
RT	Reverse transcription
STAT	Signal transducer and activator of transcription
TCID50	Median tissue culture infective dose
TLR	Toll-like receptor
TMRCA	Time to the most recent common ancestor
Tsg101	Tumour susceptibility gene 101 protein
TYK	Tyrosine kinase
UIFN	Universal interferon
UTR	Untranslated region
VIB	Viral inclusion body

Chapter 1
INTRODUCTION

1.1 History of bluetongue

Bluetongue has been known for more than 100 years and the first detailed descriptions of its clinical manifestations and pathology come from Hutcheon (1881,1902) and Spreull (1905) (Coetzee et al., 2012a; Hutcheon, 1881; Spreull, 1905). Bluetongue was likely endemic in sub-Saharan Africa for centuries but attracted little interest from the scientific community and veterinary clinicians due the lack of overt symptoms in indigenous sheep breeds. The apparent 'emergence' of a more severe form of bluetongue coincided with the introduction of susceptible Merino sheep imported from Europe into South Africa (Coetzee et al., 2012a). Due to the abundant nasal discharge and high fever in affected animals, the disease was first referred to as "epizootic catarrh" and "malarial catarrhal fever" (or simply "fever") (Spreull, 1905). These terms were later abandoned in preference of "bluetongue", despite the fact that the actual symptom of a cyanotic tongue is rarely seen in infected sheep (Spreull, 1905).

Early studies revealed that bluetongue was caused by a filterable agent found in the blood of sick animals but not necessarily associated with red blood cells. The disease did not appear to be contagious, but blood, spleen extracts or sera from viraemic animals were shown to be infectious when inoculated into naïve sheep (Spreull, 1905). Importantly, infected animals developed strong immunity and were protected against subsequent challenge with infectious material. Following these observations, in 1908 Theiler attempted to produce a vaccine for bluetongue by multiple passage of the viraemic sheep blood (Theiler, 1908). Interestingly, this first "vaccine" was used with variable success for over 40 years and it induced a degree of protection against several BTV serotypes (Coetzee et al., 2012a). In 1948, Neitz performed extensive immunological studies in sheep that for the first time proved the existence of multiple antigenically diverse types of BTV. Cross-protection assays revealed that infection with a single virus type induced protection against the same strain but was not necessary effective against heterologous strains (Howell, 1960; Howell et al., 1970). Later, Howell and colleagues defined the first 16 BTV serotypes by means of serum-neutralisation assays. This number has now expanded to 27, with the last serotype defined in 2015 (Jenckel et al., 2015).

Initially, bluetongue was thought to be confined to Africa. However, in 1943 the first confirmed outbreak of BTV occurred in Cyprus, which affected 60-70% of sheep leading

to more than 2,500 deaths across the island (Sellers, 1975). Earlier reports suggest that BTV emerged in Cyprus in the 1920s and caused significant animal losses in 1924 and 1939. At that time, the disease was known as “stomatitis” and was not associated with BTV until the agent was recognised as BTV by the Ondersrepoort Veterinary Institute in 1943 (Erasmus, 1985; Sellers, 1975). Following the Cyprus outbreak, further epidemics occurred in Israel, Pakistan and India and in 1956, BTV emerged in Southern Europe for the first time. Detection of bluetongue in 1952 in California, at that time referred to as “sore muzzle”, exposed significant potential for BTV expansion and the capability of the virus for adaptation to a variety of environmental factors (Hourrigan and Klingsporn, 1975b). Indeed, since that time, different serotypes have been isolated in all continents with the exception of Antarctica. Until recently BTV was only considered endemic in tropical and subtropical parts of the world (between latitudes 35^{o}S and 40^{o}N) and in recent years bluetongue has been spreading northwards as far as the UK and Canada (Purse et al., 2005).

1.2 Emergence of BTV-8

Since 1998, several BTV serotypes (including BTV-1, -2, -4, -9, -16) have been introduced into Europe but they were only found in the Southern countries. The situation changed after August 2006 when BTV-8 arrived in Northern Europe. The strain that caused a great number of outbreaks across the continent was unusual in several ways. Not only was it the first BTV serotype able to invade regions beyond latitude 52^{o}N but it also appeared to be remarkably virulent in the European sheep breeds (Worwa et al., 2009). After the sudden outbreak of BTV-8 in 2006, it was hoped that the virus would not survive the cold winter period. Unfortunately, the virus not only overwintered in North Europe, but when it re-emerged in 2007, it affected an even greater number of holdings, and for the first time reached the United Kingdom (Wilson and Mellor, 2009). Interestingly, this BTV-8 strain also caused symptomatic infections in cattle in some cases, and it was also later established that it could also cross the placental barrier (Backx et al., 2009; Dal Pozzo et al., 2009; Santman-Berends et al., 2010a; Santman-Berends et al., 2010b; van Schaik et al., 2008). BTV-8 caused a higher incidence of respiratory symptoms, oedema, coronitis and necrosis of the tongue in sheep than in cattle, while the latter frequently suffered from lesions of nasal mucosa, conjunctivitis and teat (Elbers et al., 2008b). While the total morbidity of sheep in the 2006 outbreak in the Netherlands was estimated to be

low, median case fatality reached over 50%. Although case fatality was over 13 times lower for cattle, in affected herds, up to 80% of cattle displayed clinical signs (Elbers et al., 2008c).

BTV-8 first emerged in Europe in the region of Maastricht in the Netherlands, bypassing all the common introduction routes previously described for other circulating BTV strains in Europe (Wilson and Mellor, 2009). The ability of the BTV-8 to spread vertically from mother to foetus, which is a phenomenon that was previously associated with tissue-culture adopted strains, raised the suspicion that sudden emergence of this novel serotype/strain was somehow linked to the use of South African 'Group B' multivalent live vaccine (Maan et al., 2008). Sequence analysis of the full genome of BTV-8NET2006/4 revealed however that the strain was not derived from the vaccine. Phylogenetic analysis of segment 2 (S2) showed close relation with a Nigerian strain of BTV-8 indicating that BTV-8NET2006/4 might have originated in sub-Saharan Africa (Maan et al., 2008). Although BTV-8 has been circulating in Africa for many decades, the route of its introduction to Northern Europe remains unclear.

1.3 *Reoviridae* and Orbiviruses

The family *Reoviridae* is composed of 15 genera of double stranded (ds) segmented RNA viruses, which infect a wide spectrum of hosts (e.g. plants, insects, fish and mammals) and include several important human and veterinary pathogens (Table 1). Viruses belonging to *Coltivirus*, *Fijivirus*, *Orbivirus*, *Oryzavirus* and *Phytoreovirus* are transmitted by insect vectors while the others are mainly spread by the faecal-oral route (Mellor, 2009).

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Table 1. Genera within *Reoviridae*.

Genus	Number of genome segments	Host	Vector
Aquareovirus	11	Molluscs, fish, Crustacea	-
Cardoreovirus	12	Crustacea	-
Coltivirus	12	Mammals (including humans)	Tick
Cypovirus	10	Insects	-
Dinovernavirus	9	Mosquitoes	-
Fijivirus	10	Plants	Planthopper
Idnoreovirus	10	Insects	-
Mimoreovirus	11	Phytoplankton	-
Mycoreovirus	11/12	Fungi	-
Orbivirus	10	Mammals (including humans), Birds	Midge, mosquito, sandfly, tick
Orthoreovirus	10	Birds, reptiles, mammals (including humans), fish	-
Oryzavirus	10	Plants	Planthopper
Phytoreovirus	12	Plants	Leafhopper
Rotavirus	11	Birds, mammals (including humans)	-
Seadornavirus	12	Mammals (including humans)	Mosquito

(Mellor, 2009; Yamanaka et al., 2014)

The name, *Reoviridae* is derived from the prototype of the family, *Reovirus*, (Respiratory enteric orphan virus) (Sabin, 1959) and encompasses viruses that share specific biochemical properties (Urbano and Urbano, 1994). Reoviruses possess non-enveloped icosahedral capsids, with or without an outer capsid layer. Their genome is segmented and made up of 9 to 12 segments coding for structural and non-structural proteins. The viruses carry their RNA-dependent RNA polymerase (RdRp) and RNA capping enzyme within the core. They replicate in the cytoplasm and produce distinct inclusion bodies involved in nascent virion assembly and morphogenesis. RNA transcription, capping and methylation occur within the core and mRNA is extruded into the cytoplasm through pores of the inner capsid. Due to their segmented genomes, genetic reassortment between homologous viruses can readily occur in co-infected cells (Urbano and Urbano, 1994).

Orbivirus, as a genus name, was suggested by Borden and colleagues as a reflection of large, doughnut shaped capsomers on the surface of these viruses (Borden et al., 1971).

The Orbiviruses are a group of arthropod-borne viruses. Twenty-two Orbiviruses are currently recognised by the International Committee on Taxonomy of Viruses (ICTV) and several tentative species are awaiting to be included in the genus (Figure 1) (Belaganahalli et al., 2015). Members of *Orbivirus* were commonly identified by high levels of serological cross-protection against conserved antigens, e.g. VP7 or cross-hybridisation of conserved genome segments (>70% identity required), conserved termini sequences and clinical presentation and host/vector range. Viruses in the genus share 21 to 72% sequence identity in conserved S3 (Mellor, 2009).

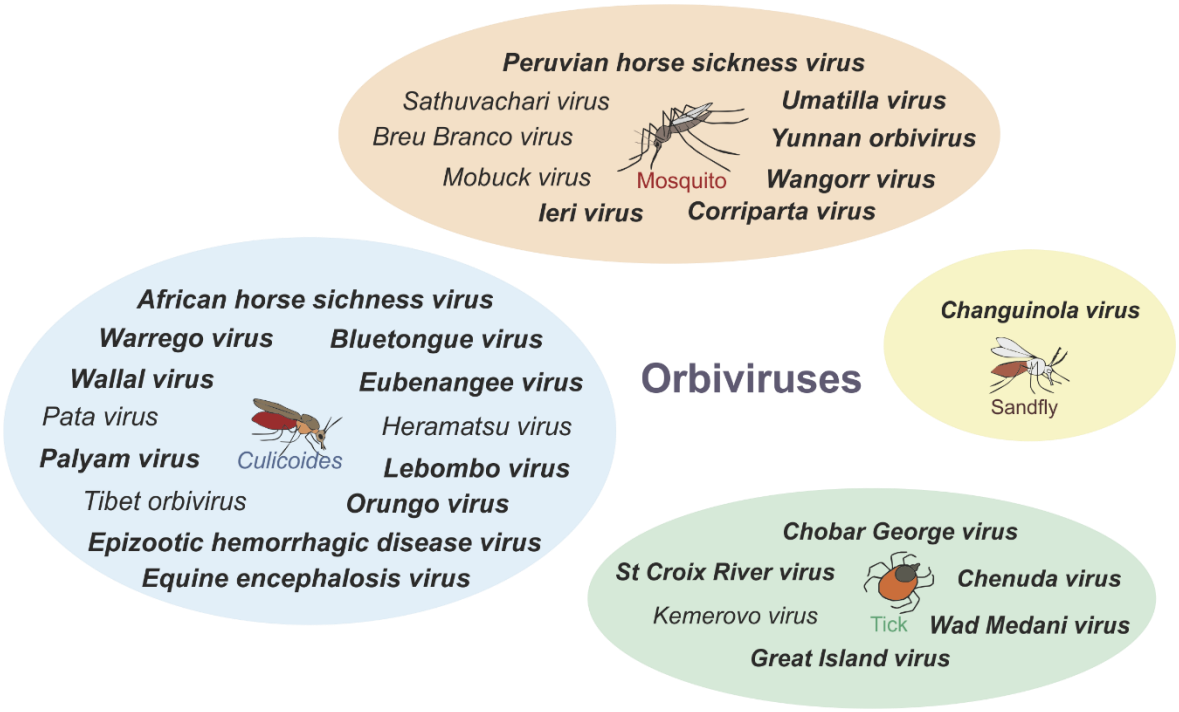

Figure 1. Orbiviruses and their vectors. ICTV-recognised orbiviruses (bold italics) and proposed *Orbivirus* species (italics) (Belaganahalli et al., 2015).

Apart from BTV, several other Orbiviruses, including African horse sickness virus (AHSV), Epizootic hemorrhagic disease virus (EHDV) and Equine encephalosis virus (EEV) cause economically important disease in domesticated and wild ruminants. Humans can also be infected by some of the species e.g. Changuinola virus, Corriparta virus, Orungo virus, Lebombo virus and Kemerovo virus (Belaganahalli et al., 2013; Brown et al., 1991; Libikova et al., 1978; Silva et al., 2014). The history of the BTV expansion and the consequences of climate change on vector expansion have given rise to concern over the increased risk of incursions of other Orbiviruses into new areas where competent vectors and susceptible hosts reside (Gale et al., 2010; MacLachlan and Guthrie, 2010). This in

turn would have an impact on wildlife populations, food production and possibly human health.

1.3.1 African horse sickness virus (AHSV)

AHSV is the cause of a haemorrhagic disease of equids (known as African horse sickness) and it is transmitted by *Culicoides* biting midges. Similar to BTV, AHS has likely been endemic in southern Africa for hundreds of years but it was first recognised during epidemics in 1719, around 60 years after introduction of horses to the area (Mellor and Hamblin, 2004). Since that time, the virus caused multiple outbreaks in South Africa, with the largest one in 1854-1855 that caused over 700,000 deaths of infected horses (Mellor and Hamblin, 2004). The disease is endemic in Africa but outbreaks have also been reported in the Middle East, Cyprus, Spain and Portugal (MacLachlan and Guthrie, 2010). To date, nine serotypes of AHSV have been recognised, two of which (AHSV-4 and AHSV-9) in Southern Europe (Howell, 1962; Wilson et al., 2009). AHSV infects zebra, which are considered a main reservoir of the virus. Zebra rarely show signs of infection while the sickness can be very severe in other equids. The virus primarily replicates in lymphoid tissues including the spleen, and also in the lungs and certain types of endothelium. The infection can be expressed as horse sickness fever, cardiac form, pulmonary form or mixed form. The latter, which is a combination of both cardiac and pulmonary symptoms, is the most common and results in 70% mortality (Laegreid et al., 1993). Modified live vaccines (MLV) against AHSV are available and immunisation with polyvalent MLV is compulsory in Southern Africa (von Teichman et al., 2010).

1.3.2 Epizootic haemorrhagic disease virus (EHDV)

EHDV is one of the Orbiviruses that are most closely related to BTV (Belaganahalli et al., 2014). The virus can infect most ruminants, however, the disease is most pronounced in wild animals including white-tailed deer. Like BTV and AHSV, EHDV is transmitted by *Culicoides* biting midges and the disease has been reported in multiple parts of the world including North America, Africa, Asia and Australia. EHD has also spread to several countries of the Mediterranean basin, but it has not been introduced into Europe yet (Savini et al., 2011). Clinical symptoms of EHD range from inapparent or mild infection to severe disease expression with widespread oedema, haemorrhage and death (Batten et al., 2011; Ruder et al., 2012). Currently there are eight serotypes of EHDV recognised

although AHDV-3 has been suggested to be included into the EHDV-1 serogroup based on sequence relatedness (Anthony et al., 2009). The difference between virulence of specific serotypes or strains in unclear. However, western strains (isolated from ruminants in the Middle East, Africa or North America) seem to be more pathogenic than the eastern strains (isolated from animals in Japan or Australia) (Anthony et al., 2009).

1.3.3 Equine encephalosis virus (EEV)

EEV is another *Culicoides*-borne *Orbivirus* associated with disease in horses. It was first isolated in South Africa in 1967 from a mare that died of an unknown illness with neurological symptoms (Viljoen and Huismans, 1989). Within 3 years from the first isolation, antibodies to EEV were found in over 75% of tested horses in South Africa (Viljoen and Huismans, 1989). All equidae are susceptible to EEV but donkeys and zebras are usually asymptomatic (Oura et al., 2012). To date, seven serotypes have been described, which may produce different disease syndromes in horses (Howell et al., 2002). In general, EEV infection is asymptomatic or very mild and rarely results in death (Oura et al., 2012). In severe cases, infection may be associated with oedema and brain swelling (encephalosis), catarrhal enteritis, cardiac failure, abortion, facial oedema or severe liver damage (Howell et al., 2002). EEV is endemic in South Africa and high EEV seroprevalence has been reported in other African countries including The Gambia, Ethiopia and Ghana (Oura et al., 2012). Additionally, a recent epidemiological study showed that the virus has been circulating in Israel since 2001, which highlighted the potential of EEV to spread to other regions where suitable hosts and vectors are found (Westcott et al., 2013)

1.4 Bluetongue virus

BTV is the *Orbivirus* prototype. It was the first virus within the genus to have a complete genome sequence determined and its structure has been studied in detail (Gouet et al., 1999; Grimes et al., 1998; Pedley et al., 1988).

BTV is a non-enveloped virus composed of an icosahedral core with two concentric protein layers containing the viral genome (19.2 kbp) and enclosed within an outer capsid. Like all members of the *Reoviridae* family, BTV possesses a segmented double stranded genome encoding for structural and non-structural proteins (Mertens et al.,

2004). Each of the 10 dsRNA segments begins and terminates with conserved hexanucleotides (positive strand sequence 5'-GUUAAA . . . ACUUAC-3') (Mertens and Sangar, 1985). The untranslated regions (UTR) overlapping the open reading frames (ORF) of all BTV proteins are essential for sequential packaging of the genome and up-regulation of viral protein translation mediated by NS1 (Boyce et al., 2012; Burkhardt et al., 2014; Sung and Roy, 2014).

Based on specific migration of the RNA molecules in polyacrylamide gels and their *in vitro* translation products, 7 structural proteins (VP1-VP7) and 4 non-structural proteins (NS1-NS4) have been assigned to the individual BTV genome segments (Table 2) (Mertens et al., 1984; Ratinier et al., 2011; Van Dijk and Huismans, 1988).

Table 2. BTV genome segments and encoded proteins.

Segment	Size (nucleotides)	Protein	Size (amino acids)	Proposed function
1	3944	VP1	1302	RNA-dependent RNA polymerase
2	2939	VP2	961	Receptor binding; cell entry
3	2772	VP3	901	Structural scaffold for VP7
4	1981	VP4	664	RNA-capping enzyme
5	1776	NS1	552	Tubule formation; up-regulation of BTV protein expression
6	1637	VP5	526	Membrane penetration; cell entry
7	1156	VP7	349	Structural; binding to receptor on insect cells
8	1125	NS2	354	VIB formation; ssRNA binding and BTV genome package
9	1049	VP6 NS4	329 77	RNA helicase IFN antagonist
10	822	NS3	229	Viral egress; IFN antagonist

Nucleotide and amino acid number correspond to BTV-8NET2006/04. (Boyce et al., 2012; Chauveau et al., 2013; Mertens and Diprose, 2004; Mertens et al., 2004; Ratinier et al.; Roy, 2005, 2008b).

The outer capsid of BTV is composed of two proteins, VP2 and VP5, responsible for attachment and penetration of the host cell membrane. Cryo-EM studies of BTV structure revealed that VP2 forms sail-shaped spikes on the surface of the virion, that

protrude away from more internally located globular VP5 molecules (Hewat et al., 1992a). Enclosed in the outer shell of the virus is the double-layered core. The intermediate layer is formed by VP7 trimers arranged as a T=13 icosahedral lattice covering the subcore. The subcore is composed of 120 copies of VP3 and it is sufficiently stable to maintain its structure even in the absence of the other structural proteins (Stuart and Grimes, 2006). The last three structural proteins, VP1, VP4 and VP6 are located inside the core in the complexes located at the five-fold symmetry axes of the BTV particle (Grimes et al., 1998).

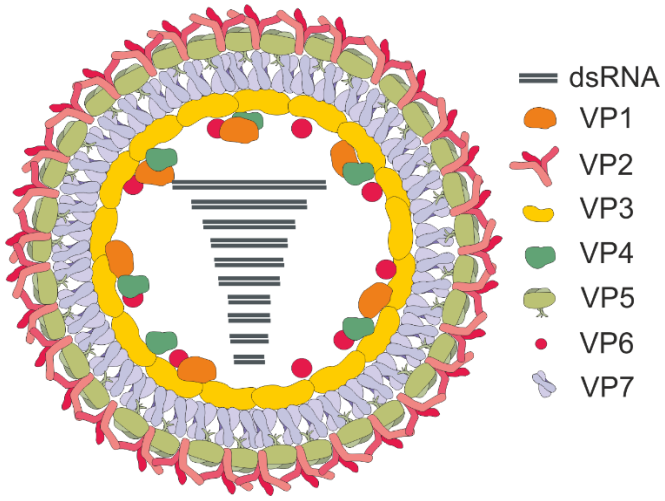

Figure 2. Schematic presentation of a BTV particle. BTV viron is assembled in three layer. The outermost capsid is formed by VP2 and VP5 proteins. The intermediate layer (core) is composed of VP7 trimers. VP3 layer (subcore) encloses the replication protein complex (VP1, VP4, VP6) and dsRNA.

1.5 BTV replication cycle

BTV binds the host cell using specific cellular factor(s) and undergoes receptor mediated endocytosis via clathrin-coated vesicles (Forzan et al., 2007). The initial steps are mediated by VP2 and VP5, which can then be detected in the early endosome (Eaton et al., 1990). Both proteins are rapidly dissociated from the virus surface and only the cores are released from the endosome into the cytoplasm. VP5 acts as a fusion protein, which, under low pH, undergoes conformational change allowing the release of the outer capsid from the VP7 layer (Forzan et al., 2004; Zhang et al., 2010).

Transcription and replication of the viral genome occurs inside the core in the host cell cytoplasm. Viral dsRNA never leaves the core and therefore it is believed to be protected from sensing by the cellular pattern recognition receptors and from destruction by cellular nucleases (Diprose et al., 2002). The complex of VP1, VP4 and VP6 acts as the viral transcriptional machinery (Mertens and Diprose, 2004). VP6 facilitates unwinding and/or separation of the two strands of each genomic segment (Stauber et al., 1997). VP1 acts as an RNA-dependent RNA polymerase (RdRp) and inside the core, it utilises the negative genome strand to transcribe viral mRNA (Boyce et al., 2004; Urakawa et al., 1989). VP4 synthesizes a methylated cap at the 5' terminus of each mRNA molecule (Martinez-Costas et al., 1998; Ramadevi et al., 1998) and the capped mRNA is then extruded through the pores in the capsid into the cytoplasm (Diprose et al., 2001; Verwoerd and Huismans, 1972). Positive strand RNA also acts as a template for synthesis of new negative strands inside the newly formed viral particles (Boyce et al., 2004). In the cytoplasm, BTV mRNA is translated by the host cell translational machinery and the nascent virions are sequentially assembled. Translation of BTV proteins starts soon after host cell entry. At 8 hours post-infection, viral proteins become the dominant fraction in the cytosol indicating that translation of host cell mRNA is effectively replaced by viral protein synthesis (Huismans, 1979). Upregulation of viral protein translation is facilitated by NS1 that has been shown to selectively increase the rate of expression of specific BTV genes (Boyce et al., 2012). The subcores and cores are formed in viral inclusion bodies (VIB) and the outer proteins are recruited as the new virions exit VIBs (Brookes et al., 1993; Kar et al., 2007). The mode of BTV egress varies with the time of infection and host cell type. In insect cells, the virus exits by budding and causes no cytopathic effect, while in mammalian cells the virions are extruded through the membrane, which consequently

leads to cell lysis (Hyatt et al., 1989; Owens et al., 2004). The trafficking and exit of the new BTV particles are mediated through NS3 interaction with a cellular protein component of the ESCRT-I (endosomal sorting complexes required for transport-I) complex, Tsg101 (Celma and Roy, 2009). Although direct cell-to cell spread has been suggested for BTV, most particles are released from mammalian cells by cell lysis (Bhattacharya and Roy, 2008). Infection of neighbouring cells can be inhibited by induction of interferon (IFN)-related antiviral factors. Viral NS4 and NS3 have been implicated in BTV counteraction of host IFN response (Chauveau et al., 2013; Ratinier et al., 2011). Other sections in this chapter will describe the roles of specific BTV proteins in viral replication and in the interactions with the host cells.

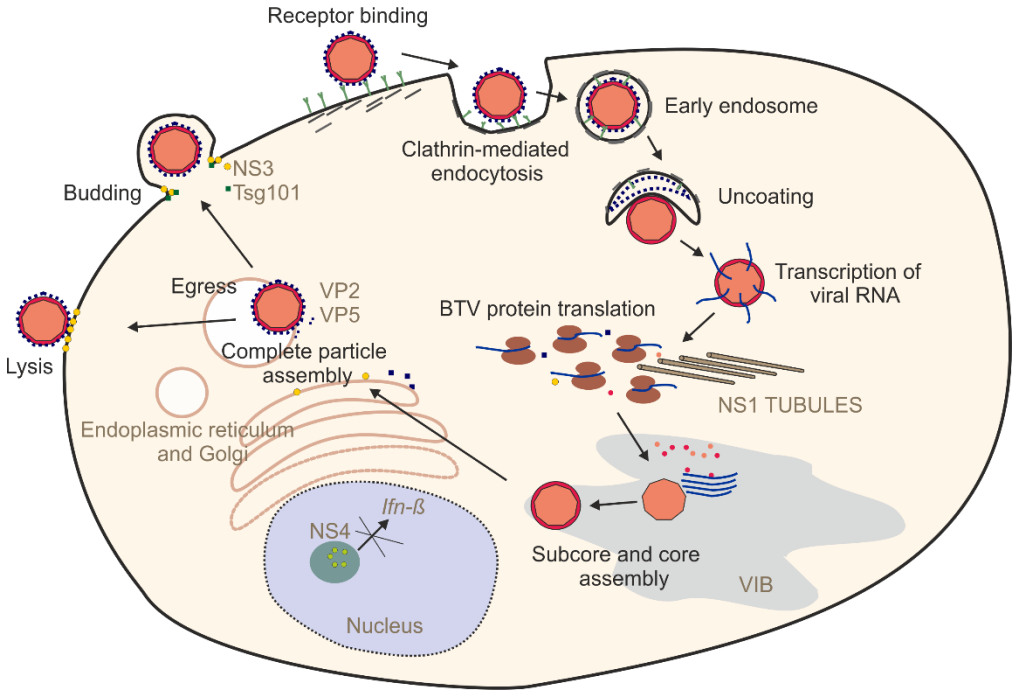

Figure 3. BTV replication cycle. BTV enters the cell by endocytosis in clathrin-coated pits. Acidification of early endosomes causes VP5 mediated membrane fusion. The outer capsid is dissociated from the particle and the core enters the cell cytoplasm. Inside the active core, transcription of viral RNA takes place and the ssRNA is extruded through the pores in the VP3 layer. BTV protein translation is performed via host cell translational machinery and viral NS1 is involved in up-regulation of BTV gene expression. Subcore and core assembly takes place in viral inclusion bodies (VIB) and it is facilitated by interactions with NS2. NS4 is transported to the cell nucleus and localises in the nucleolus where it interferes with IFN synthesis. Complete particles are formed by core association with VP2 and VP5. NS3 is involved in trafficking and egress of mature particles either by budding (through interactions with Tsg101) or by cell lysis (Mertens et al., 2004; Roy, 2008b).

1.6 BTV proteins

1.6.1 VP2 and VP5

VP2 is an outer capsid protein that forms trimers on the surface of the BTV particle (Hewat et al., 1992a; Zhang et al., 2010). The protein has been shown to elicit neutralising antibodies and it determines the various BTV serotypes (Mertens et al., 1989; Purdy et al., 1985). VP2 is the most variable among the BTV proteins with an inter-serotype amino acid sequence variation ranging from 23 to 73%. Therefore, there is no significant cross-protection between antibodies elicited against different serotypes (Maan et al., 2007a). Only two regions of VP2 are relatively conserved (amino acids 338-379 and 946-961) and the specific roles of these residues in BTV infection has not been explored further (Maan et al., 2007a).

Early studies of VP2 interactions with host cells concentrated on the ability of BTV to bind erythrocytes and causing haemagglutination in a variety of mammalian species (Eaton and Crameri, 1989) (Cowley and Gorman, 1987; Hassan and Roy, 1999; van der Walt, 1980). Consequently, BTV was shown to bind to glycophorins of human, porcine, equine and ovine origin. The binding was inhibited by addition of external glycophorins and by pre-treatment of erythrocytes with *V.cholerae* neuraminidase (Eaton and Crameri, 1989). Eaton and colleagues suggested that BTV possessed at least two sites for attachment to different glycophorin residues in human (or porcine) and ovine erythrocytes (Eaton and Crameri, 1989). Experiments using tagged recombinant VP2 protein demonstrated that VP2 was responsible for BTV binding to glycophorin A on the surface of erythrocytes and mediated virus entry into mammalian cells (Hassan and Roy, 1999). Treatment of L929 cells with neuraminidase or treatment with sodium periodate reduced VP2 binding, confirming that VP2 bound to a cellular receptor(s) rich in carbohydrate moieties. Recently, cryo-EM studies showed that VP2 possesses a sialic acid binding region located in its hub domain, which is one of two sites suggested to interact with cell surface receptors (Zhang et al., 2010). However, the presence of another putative receptor-binding site at the VP2 tip domain strongly suggests that BTV utilizes another cellular factor for cell entry purposes. Furthermore, wheat germ competition assay studies showed that in the presence of wheat germ protein, which block sialic acid sites on the cell surface, BTV infectivity was reduced but not entirely

abolished (Zhang et al., 2010). Altogether, these studies showed that apart from sialic acid glycoprotein, another receptor must be involved in BTV attachment to the host surface (Hassan and Roy, 1999; Zhang et al., 2010).

VP2 has also been shown to participate in BTV egress from the infected cells. Yeast two-hybrid screening of a BTV protein library showed that VP2 interacted specifically with the C-terminal domain of NS3. Additionally, VP2 co-localized with NS3 in the Golgi apparatus, along the vesicle exocytic route and at the plasma membrane. These observations suggested that NS3 bridged the mature BTV particle through the VP2 and facilitated its transport to the cell surface (Beaton et al., 2002). The N-terminal of VP2 was also shown to associate with vimentin, and disruption of the vimentin network led to accumulation of intracellular BTV particles, suggesting that VP2-vimentin interactions play a role in virus release from infected cells (Bhattacharya et al., 2007).

VP5 is the smaller of the outer capsid proteins and forms globular trimer complexes connected with the hub domains of the neighbouring VP2 trimers (Zhang et al., 2010). Cryo-EM studies showed that VP5 is composed of two domains separated by a flexible hinge. The N-terminal domain (amino acids 1-240) contains a coiled-coil structure while the C-terminal (amino acids 260-526) forms a globular domain (Hassan et al., 2001; Zhang et al., 2010). The predicted model of VP5 shows that the N-terminal region is composed of two amphipathic helices followed by a stretch of hydrophobic residues, which have been demonstrated to play a role in membrane destabilization and cytotoxicity (Forzan et al., 2004; Hassan et al., 2001). VP5 can induce cell-cell fusion and its membrane penetration properties are dependent on low pH, which is likely to be responsible for the conformational changes within the VP2 protein, enabling the VP5 amphipathic helices to interact freely with the membrane of the endosome (Forzan et al., 2004). Low pH has also been suggested to cause loosening of the interactions between the outer capsid proteins and the viral core, facilitating the release of transcriptionally active particles into the cytoplasm. In addition, VP5 has been shown to interact with lipid rafts. Disruption of the rafts by exclusion of cholesterol from the cells leads to a significant decrease in BTV titres, confirming the roles of lipid rafts in the BTV replication cycle (Bhattacharya and Roy, 2008). Additional experiments showed that VP5 directly interacted with NS3. This prompted the hypothesis that lipid rafts formed a scaffold for assembling the viral particles and that NS3 stabilised both VP5 and VP2 for

the final particle assembly (Bhattacharya and Roy, 2008). Interestingly, the C-terminal of VP5 contains a conserved WHXL motif that is also found in synaptotagmin-1 (Syt1), a SNARE regulatory protein. Mutation of this amino acid stretch leads to abrogation of VP5 localization to the plasma membrane. VP5 is therefore likely to be specifically targeted to host cell plasma membranes through membrane-docking signals of the SNARE proteins (Bhattacharya and Roy, 2008). Additionally, due to its co-localisation with "pore-like structures" present at the cellular junctions of infected cells, VP5 has been hypothesised to participate in cell-to-cell transfer between infected and uninfected cells (Bhattacharya and Roy, 2008). This hypothesis, however, has yet to be confirmed.

1.6.2 VP3 and VP7

The core of BTV is enclosed in two protein layers. The innermost layer is composed of 120 copies of VP3 which is a 110 kDa protein encoded by genome S3. The VP3 shell serves as a scaffold for attachment of VP7 and together they form stable core-like particles (CLP) (Grimes et al., 1998; Tanaka et al., 1995). X-ray crystallography studies demonstrated that monomers of VP3 assemble to form decamers, each decamer is formed by two sets of 5 proteins with different conformations ('A' and 'B' forms) (Grimes et al., 1998; Grimes et al., 1997). These VP3 decamers, which resemble a dish-shaped structure, are likely to be the first assembly intermediates in formation of new virions and are subsequently bound by VP1 and VP4 (Kar et al., 2004; Nason et al., 2004). Studies using GFP-tagged VP3 showed that when expressed together with VP7, VP3 is present primarily in VIBs, the site of viral replication and assembly (Kar et al., 2005). VP3 is composed of 901 amino acid residues structurally organised into three domains, 'apical' (residues 298-587), 'carapace' (residues 7-297, 588-698 and 855-901) and 'dimerisation domain' (699-854) (Grimes et al., 1998). Several studies have investigated the roles of specific domains in assembly of the core particles (Kar et al., 2004; Kar et al., 2005; Tanaka et al., 1995). It has been shown that, whereas deletion of the C-terminal of VP3 did not affect VP3-VP3 nor VP3-VP7 binding, the N-terminal sequence of VP3 was essential for interactions with other BTV proteins, particularly VP7 (Kar et al., 2004). The dimerization domain is crucial for the ability of the new forming core to bind viral RNA (Kar et al., 2004).

VP7 is 38 kDa protein arranged into 260 trimers that form the outer surface of the BTV core (Basak et al., 1997; Basak et al., 1992; Grimes et al., 1998). Structural studies of VP7 showed that each of its monomers is composed of two separate domains. The "upper" domain of one molecule interacts with the "lower" domain of an adjacent monomer by hydrophobic and hydrogen bond interactions. The upper domain consists of a central amino acid chain (amino acid 121-249) and it is folded into an antiparallel β-sandwich. The upper domains of a trimer form its head region, which protrudes outwards from the viral core. The larger, lower domain is composed of the N-terminal (amino acids 1-120) and the C-terminal (amino acids 250-349) of the protein, which together form 9 α-helices and extended loops (Basak et al., 1997; Roy, 2005). The lower domain interacts with VP3 and the sides of other VP7 trimers (Grimes et al., 1995). VP7 contains two cleavage sites; before Gly127 and Tyr250. Although the importance of these sites in BTV infection has not been shown, Basak and colleagues speculated that the cleavage and the resulting conformational change in VP7 could occur prior to membrane penetration and would facilitate BTV entry into insect cells (Basak et al., 1997). Indeed, another study showed that the core particles of BTV are 103 times more infective to *Culicodes* KC cells than to the mammalian cell line BHK-21, suggesting that the surface proteins of the core are directly involved in viral entry in *Culicoides* cells (Mertens et al., 1996). Therefore, distinct BTV proteins, as well as receptor molecules, appear to be involved in BTV entry into mammalian and insect cells (Xu et al., 1997).

VP7 contains a conserved arginine-glycine-aspartate (RGD) motif located in the exposed part of the upper domain (amino acid residues 168-170) (Grimes et al., 1995). This tripeptide motif is a common recognition sequence for integrins and, as such, could be involved in BTV binding to the cell membrane of insect cells (Basak et al., 1997; Tan et al., 2001). Using core-like particles (CLP) composed of VP3 and VP7 that retain the three-dimensional structure of the BTV core, Tan and colleagues explored how mutations in the RGD motif affect CLP binding to *Culicoides* cells. CLPs with mutated RGD regions showed reduced binding to the surface of insect cells. Additionally, monoclonal antibodies raised against the region containing the RGD tripeptide were able to compete for binding to CLPs, showing that this motif in VP7 is exposed and easily accessible for attachment to external molecules (Tan et al., 2001). Altogether, these data showed that VP7 was a likely candidate for receptor binding protein in insect cells.

1.6.3 VP1, VP4 and VP6

The BTV core contains three minor proteins, VP1, VP4 and VP6, which are essential for viral RNA synthesis and transcription, and together form replication complexes associated with each of the 10 genome segments (Roy, 2008b). Double stranded RNA never leaves the core and thus remains protected from the cell's antiviral surveillance machinery (Mertens and Diprose, 2004).

The largest of the BTV proteins, VP1 (149.5 kDa) is an RNA-dependent RNA polymerase (RdRp) which is present in approximately 12 copies per viral particle (Grimes et al., 1998; Urakawa et al., 1989). VP1 acts as a replicase and in the absence of other BTV proteins, it can initiate and synthesise minus strand RNA *de novo* (Boyce et al., 2004; Matsuo and Roy, 2011). At 37^{o}C, recombinant VP1 remains active for at least 23 h. However, replicase activity is relatively low suggesting that the efficiency of replication might be modulated by other BTV proteins (Boyce et al., 2004). Further experiments showed that the replication efficiency of VP1 was enhanced by the presence of 5' cap structure in the template ssRNA (Matsuo and Roy, 2011). Interestingly, the activity of VP1 is not affected by removal of conserved 3' end hexanucleotides, and the enzyme can synthesise dsRNA from genomic segments of other members of the *Reoviridae* (Boyce et al., 2004; Matsuo and Roy, 2011). It is therefore possible that VP1 sequence specificity and preference for viral over foreign templates is conferred by secondary structures of ssRNA (Matsuo and Roy, 2011).

The structure of VP1 has not been resolved to date. However, 3D models of BTV-1 VP1, based on the structure of known RdRp molecules, provided insights on the structural organisation of this protein (Wehrfritz et al., 2007). According to the model, VP1 is composed of the N-terminal domain (amino acids 1-373), the polymerase domain (581-880) and the C-terminal domain. Replicase activity has been mapped to the polymerase domain but it requires the two other domains for its activity (Wehrfritz et al., 2007). The polymerase domain of VP1 possesses a 'right hand' structure with fingers (581-632, 672-731), palm (633-671, 732-810) and thumb (811-880) subdomains and it is responsible for NTP binding. At the core of the palm subdomain there is a GDD motif (763-765) which serves as a catalytic site of the enzyme (Wehrfritz et al., 2007).

Before translation, newly synthesised BTV transcripts need to be capped and this process takes place inside the core. This function is provided entirely by VP4, which has an RNA triphosphatase, guanyltransferase, guanine-N7-methyltransferase and 2'-O-methyltransferaze activities (Le Blois et al., 1992; Martinez-Costas et al., 1998; Ramadevi and Roy, 1998; Roy, 2008a). The resolved atomic structure of BTV-10 VP4 allowed mapping of these catalytic activities to specific regions of the protein (Sutton et al., 2007). In the first step in cap formation, hydrolysis of the 5'triphosphate to diphosphate is catalysed by RtPase which likely resides within the most conserved C-terminal domain of VP4 (GT-domain). The GT-domain (C-terminal 135 amino acids) is also proposed to contain the GTPase activity responsible for addition of GMP via a 5'-5' triphosphate linkage to the diphosphate terminus. The next step in cap formation, i.e. transfer of a methyl group to the N7 position, is catalysed by N7MTase. The N7MTase domain is split between amino acid residues 110-154 and 370-509, between which the 2'OMTase domain is inserted (amino acid residues 175-377). 2'OMTase catalyses methylation of a 2'hydroxyl group in the ribose of the 5' terminal nucleotide. The active site of 2'OMTase has been mapped to the KDKE tetrad, encompassing Lys178, Asp265, Lys306 and Glu335 (Sutton et al., 2007). Both VP4 methyltrasferases use AdoMet as a substrate and *in vitro* experiments confirmed that in the presence of AdoMet, VP4 could modify BTV mRNA to form a fully methylated cap structure (Ramadevi and Roy, 1998). Interestingly, VP4 possesses another domain at its N-terminal (first 108 amino acids) which contains a kinase fold but lacks some of the typical features of kinases (Sutton et al., 2007). This domain (termed KL) was proposed to facilitate protein-protein interactions, possibly with VP1 or VP3 during core assembly (Sutton et al., 2007).

The smallest enzymatic protein found in the core is VP6 (35.7 kDa), a viral helicase that acts early in BTV replication (Matsuo and Roy, 2009). It possesses nucleic acid-binding sites and can act as an RNA-dependent ATPase (Calvo-Pinilla et al., 2009a; Roy et al., 1990; Stauber et al., 1997). *In vitro*, in the presence of ATP and magnesium ions, VP6 can bind blunt-ended dsRNA as well as duplexes with 3' or 5' overhangs, and it is capable of unwinding double-stranded RNA molecules. Hence, it likely assists in the transcription process by unwinding the RNA duplex ahead of active VP1 replicase, or by separating the newly synthesised strand from the template after transcription (Roy, 2008a; Stauber et al., 1997). The nucleic acid binding properties of VP6 have been partially mapped to two

regions located within amino acid residues 181- 212 and at the C-terminal (Hayama and Li, 1994). A study by Kar and colleagues showed that two conserved motifs played roles in VP6 ability to unwind the RNA duplex (Kar and Roy, 2003). An AxxGxGK110V motif is essential for ATPase activity and Lys110Asn mutation abrogated the ability of VP6 to bind ATP. An Arg205Gln mutant in the arginine-rich RxGRxxR motif also showed reduced ATPase activity, but ATP binding was not affected. Additionally, the latter mutant was not able to bind RNA, which confirmed that ATPase function was directly related to RNA-binding activity (Kar and Roy, 2003).

1.6.4 NS1

BTV encodes four non-structural proteins. NS1, the largest of these proteins, forms tubules in the cytoplasm of BTV infected cells (Hewat et al., 1992b). NS1 is the most abundant viral protein synthesised in the infected cells, constituting 25% of all viral proteins (Van Dijk and Huismans, 1988). Multimers of NS1 form helically coiled ribbons 68 nm in diameter and can reach 1000 nm in length (Huismans and Els, 1979). The carboxy terminus is required for tubule formation and deletion of 10 C-terminal amino acids abrogates its function (Monastyrskaya et al., 1995). Mutations of two cysteine residues positioned at 337 and 340 in the hydrophilic region of the protein lead to products that are polymerised into ribbon-like structures but do not form clearly defined tubules (Monastyrskaya et al., 1994). Tubules first appear 2-4 h post infection at approximately the same time as synthesis of other BTV proteins can be observed, but no virus progeny have yet formed. While, at the early stages of infection, tubules are mostly associated with viral inclusion bodies, later they can be seen dispersed across the host cell cytoplasm (Huismans and Els, 1979). Cryo-electron microscopy studies showed that tubules could be associated with intermediate filaments of the infected cells (Hewat et al., 1992b). NS1 is highly conserved among different serotypes but its function has not been fully elucidated.

NS1 is involved in cellular pathogenicity and disruption of tubule formation leads to a change in the mode of BTV exit from infected cells (Owens et al., 2004). Using antibodies that interfered with NS1 tubule formation, Owens *et al*. induced a shift in mammalian infected cells from lytic release of virions, to egress via budding through the cell membrane (Owens et al., 2004). Viral replication was not affected but little virus-induced

CPE was observed compared to when the tubules were intact. Since NS1 tubules are also abundant in insect cells, which do not display CPE when infected with BTV, another factor has been proposed to affect viral egress via budding from the cell membrane (Owens et al., 2004). Another BTV protein, NS3, is abundantly expressed in infected invertebrate cells where the NS1:NS3 ratio is much higher compared to what is observed in mammalian cells. NS3 has been shown to facilitate non-lytic release of virions via the calpactin dependant exocytic pathway (Beaton et al., 2002). Owens and colleagues hypothesised that NS1, in conjunction with NS3, might play a role in the different mechanisms followed by BTV to exit the infected cell (Owens et al., 2004). High NS1 levels relative to NS3 (as seen in mammalian cells) would favour accumulation of BTV progeny in the cytoplasm, leading to cell lysis and virion release, while low NS1:NS3 ratio would lead to BTV egress via a budding mechanism (Owens et al., 2004).

Until recently, the mechanism by which BTV mRNA competes with the host mRNA for the protein translation machinery has been unclear. Boyce and colleagues showed that NS1 is sufficient to preferentially upregulate BTV translation (Boyce et al., 2012). The authors used a reporter RNA composed of a GFP ORF incorporated within the sequence of S10 to examine the effect of individual BTV segments on gene expression (Boyce et al., 2012). The increase of fluorescence was observed only when cells were transfected with the reporter in the presence of NS1. The UTRs of viral RNA were shown to be sufficient for NS1 induced up-regulation of protein expression. However, the relative level of expression varied between individual BTV genes, suggesting that specific UTR sequences dictate the amount of protein synthesis from individual genes (Boyce et al., 2012).

1.6.5 NS2

NS2 is a major component of VIBs, large perinuclear structures that increase in size and density as infection progresses (Thomas et al., 1990). VIBs can be observed within four hours and reach their peak at about 18 hours post infection (h.p.i.) (Brookes et al., 1993). VIBs are the sites of viral protein synthesis and assembly and contain newly formed virions at different stages of morphogenesis (Brookes et al., 1993). By expressing NS2 in insect cells, Thomas *et al.* showed that NS2 was associated with VIB but not with free

virions, and that it could form inclusion bodies within the cells even if expressed independently of the other BTV proteins (Thomas et al., 1990).

NS2 has a strong affinity for single stranded RNA but it does not bind double stranded RNA (Huismans et al., 1987b; Lymperopoulos et al., 2006; Thomas et al., 1990). The protein has been reported to interact with ssRNA in a non-specific manner (Huismans et al., 1987b; Taraporewala et al., 2001). However, recent studies showed that NS2 preferentially binds to BTV-specific RNA (Lymperopoulos et al., 2006; Lymperopoulos et al., 2003). RNA binding regions are located at amino acid residues 2-11, 153-166, and 274-286, and each of these domains differ in their affinity for ssRNA (Fillmore et al., 2002). Lymperopoulos *et al.* suggested that NS2 might have particular domains solely responsible for binding to BTV RNA as well as sites that can bind ssRNA non-specifically (Lymperopoulos et al., 2003). The RNA sequences recognised by NS2 lie within the coding region and the protein-RNA interactions are likely due to the secondary structure of RNA rather than the primary sequence (Lymperopoulos et al., 2003). These secondary structures have been mapped for four BTV-10 segments, to nucleotide regions 901-1352 for S5, 721-861 for S8, 1-273 for S9 and 99-170 for S10 (Lymperopoulos et al., 2006; Lymperopoulos et al., 2003).

In addition to high affinity for ssRNA, NS2 has the ability to initiate BTV core formation by interacting with VP3 and indirectly recruiting VP7 (Kar et al., 2007). It has also been shown to bind VP1, the component of the transcriptase complex (Modrof et al., 2005). Together, these observations suggest that in VIBs, NS2 recruits BTV ssRNA that directly interacts with the VP1, VP4, and VP6 complexes, which are then encapsulated by VP3 and VP7 to form the viral core. The synthesis of the second RNA strand would then occur inside the previously assembled core (Lymperopoulos et al., 2003).

NS2 is the only BTV protein that is phosphorylated, a process that can be mediated *in vitro* by CK2 kinase (Modrof et al., 2005). Phosphorylation has been mapped to two serine residues located at the C-terminal at positions 249 and 259. Modrof and colleagues demonstrated that non-phosphorylated NS2 is still able to interact with RNA with equivalent efficiency to wild-type NS2. Phosphorylation, however, was essential for VIB formation as mutation of the two serine residues into alanine caused dispersion of NS2 throughout the cytoplasm. Moreover, BTV infection of BHK-21 cells expressing the

non-phosphorylated form of NS2 did not result in VIB formation (Modrof et al., 2005). NS2 can bind nucleotides and possesses nucleotidyl phosphatase. The ability to hydrolyse NTPs has been suggested to aid energy generation for transport of ssRNA and its packaging inside the viral core (Taraporewala et al., 2001).

1.6.6 NS3

NS3 is a membrane protein with two transmembrane domains within amino acid residues 117-140 and 162-183, which are spanned by a longer N-terminal domain and a short C-terminal domain (Bansal et al., 1998; Beaton et al., 2002). Both N-terminal and C-terminal domains are located in the cytosol, and the extracellular domain located in-between contains the only glycosylation site (asparagine at position 150) (Beaton et al., 2002). Two conserved late domains have been identified within the N-terminal cytoplasmic domain of NS3. Late domains are commonly found in enveloped viruses that utilise budding for virion release (Celma and Roy, 2009; Freed, 2002). NS3A is a shorter variant of NS3 that lacks the 13-N terminal amino acid residues and it is translated from a second initiation codon at position 14 within the ORF of NS3.

Expression of NS3/NS3A varies between mammalian and insect cells with much higher NS3A levels in the latter (Guirakhoo et al., 1995). Guirakhoo and colleagues observed that a BTV-10 strain adapted to C6/36 cells did not cause CPE in this cell line. Additionally, the concentration of viral particles recovered from the medium of infected insect cells was much higher than from BHK-21 cells infected with the parental strain (Guirakhoo et al., 1995). As mentioned above, two different modes of BTV egress have been demonstrated. The lytic cycle, with distinctive CPE is typically seen in mammalian cells, whereas the virus is released from insect cells through budding from the cell membrane (Celma and Roy, 2009; Guirakhoo et al., 1995). These observations prompted speculations that NS3/NS3A plays a crucial role in viral egress in its vector.

Yeast two-hybrid approaches revealed interactions between NS3 and the mammalian protein A100A10/p11, which is a light chain component of the calpactin complex involved in trafficking and cellular exocytosis (Beaton et al., 2002). Binding of A100A10/p11 is dependent on the first 13 amino acid residues of NS3 (absent in NS3A), forming a putative amphipathic helix. The same study showed that, whereas the N-terminal portion of NS3 is involved in the interaction with A100A10/p11, the C-terminal

binds to VP2 forming a bridge between mature the virion and the exocytic machinery (Beaton et al., 2002). These data suggest that NS3 (but not NS3A) acts as an intermediate that facilitates non-lytic release of mature virions by utilising a common cellular pathway. These results are further substantiated by the fact that BTV NS3 can recruit tumour susceptibility gene 101 protein (Tsg101), a member of the ESCRT-1 complex involved in protein sorting, formation of multi-vesicular bodies and the last stages of membrane fission (Wirblich et al., 2006). Specifically, the late domain motif of NS3, PSAP, was shown to interact directly with Tsg101 *in vitro*, and with the protein's homologue in the Drosophila infection model (Wirblich et al., 2006). The ability to bind A100A10/p11 and Tsg101 is likely to be more important in establishing persistent infection in the vector than in the infection of the mammalian host (Roy, 2008b; Wirblich et al., 2006). The second late domain motif in NS3, PPRY, is located downstream of PSAP and separated by just one amino acid residue. The PPxY motif acts as a ubiquitination substrate of enzymes of the Neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) family and is critical for budding and release of retroviruses from the host cell (Ingham et al., 2004). NEDD4 proteins regulate multiple cellular ubiquitin-mediated processes including targeting of proteins for proteasomal degradation, protein sorting and transport via endocytic pathways. (Ingham et al., 2004). PPRY is highly conserved among all serotypes of BTV (Bhattacharya et al., 2015). When this motif was mutated into AARA, distribution of BTV in ovine PT cells changed and the virions were no longer found inside vesicles (as seen for wild type BTV), but were found free inside the cytosol in the areas surrounding the vesicles (Bhattacharya et al., 2015). Additionally, virus mutants showed decreased yields in BSR and PT cells and a significant reduction specifically in the titres of BTV released into cell culture medium. It is possible that the PPRY motif plays a role in non-lytic exit of BTV, similar to the manner of enveloped virus egress, and facilitate cell-to-cell spread in the early stages of infection (Bhattacharya et al., 2015). Both late domain motifs, PPxY and PxAP, are well conserved amongst many species of *Culicoides*-borne Orbiviruses such as AHSV, EHDV, Pata virus, Changuinola virus, Chuzan virus and Lebombo virus, while others (e.g. Orungo virus and EEV) possess only one complete late domain (Belaganahalli et al., 2015). Interestingly, mosquito-borne Orbiviruses such as Peruvian horse sickness virus and Yunnan virus carry no late domain motifs in their NS3 sequence, and these viruses cause cell death in mosquito

C6/36 cells (Attoui et al., 2009). The role of late domains in Orbiviruses other than BTV have not been described to date.

A recent study showed that expression of NS3, and NS3A in particular, is essential for release of BTV from *Culicoides* cells. Mutations of either the first or the second start codon in S10 dramatically reduced the titres of BTV recovered from culture medium, while the amount of cell-associated virus remained similar to wild type BTV. Interestingly, mutation of the second methionine did not affect viral release from the mammalian cells (van Gennip et al., 2014). The authors concluded that both NS3 and NS3A were necessary for BTV egress from KC cells, while only NS3 was essential in the mammalian host. However, the specific role of the second methionine residue in NS3 binding to A100A10/p11 (and not related to initiation of NS3A translation) was not discussed (Beaton et al., 2002; van Gennip et al., 2014).

Interaction of NS3 with VP2 mediates virus release from the host cell. *In vitro* studies showed that both proteins co-localize in the Golgi apparatus, along the exocytic pathway and plasma membrane of the host cell. These observations suggest that interaction of the two proteins plays a role in viral egress. Based on sequence comparison of NS3 proteins of different Orbiviruses, Celma and Roy identified a conserved region in the C-terminal region of NS3 (amino acid residues 196-202) likely to be involved in NS3-VP2 binding. Using a series of NS3 mutants with Ala substitutions in residues either 196-198 and/or 201-202 or a STOP codon in position 212, the authors examined the importance of specific amino acids in the C-terminal (Celma and Roy, 2009). The mutants co-localised with VP2 in the same manner as the wild type proteins. However, they did not bind VP2 in pull-down assays, which suggested that the interaction between the proteins was disrupted by these mutations. It was impossible to rescue viruses containing mutations in NS3 residues 201-202 using reverse genetics, and the other mutant viruses that were rescued, showed decreased growth compared to the wild type controls. Additionally, one of the virus mutant containing a premature stop codon resulting in the deletion of the last 18 amino acid residues (BTVCT4) was shown to be released from BSR cells 4 times less efficiently than the control virus.

The properties of NS3 described above suggest that NS3 is essential for BTV egress and that it interacts with both viral and host cellular factors to facilitate trafficking of the

nascent particles (Roy, 2008b). Additionally, the protein was shown to inhibit the IFN-β promoter by acting on the IFN expression pathway downstream of RIG-I and upsteam of IKKε (Chauveau et al., 2013). Surprisingly however, recent work by van Gennip and colleagues suggested that NS3 was not essential for BTV replication (van Gennip et al., 2014). The authors based their conclusion of the fact that they were able to rescue and propagate BTV carrying mutations in the first two start codons in the NS3 ORF. The mutants produced CPE in BSR cells albeit it was delayed in mutAUG1 and mutAUG1+2-infected monolayers. The same study also showed that insertion of 4 bp restriction sites at amino acid positions 56 or 88 that led to expression of truncated forms of NS3 abrogated CPE formation until revertant mutants emerged in subsequent passages (van Gennip et al., 2014). Moreover, mutant viruses lacking a stretch of amino acids between the first and the second AUG of NS3 ORF (the region interacting with A100A10/p11) were found not to be viable (Feenstra et al., 2014). Further work is therefore necessary to clarify the importance of S10/NS3 in BTV replication *in vitro*.

1.6.7 NS4

Until recently, it was believed that the BTV genome is monocistronic (Firth, 2008; Ratinier et al., 2011). Bioinformatics analysis of S9, however, revealed the presence of a second ORF located at +1 position with respect to the VP6 ORF. This ORF encodes a protein of 77-79 amino acids, which is highly conserved among different BTV serotypes (Ratinier et al., 2011). NS4 contains 11 basic amino acids at the N-terminal domain, shown to be involved in nuclear trafficking of the protein. Due to the presence of a leucine zipper at the C-terminal, the protein was suggested to have nucleic acid binding properties. NS4 has been shown to be expressed in BTV-infected cells both *in vitro* and *in vivo*. The protein localises in the nucleolus and its presence in the infected cells can be detected as early as 2 hours post infection. Ratinier and colleagues rescued, by reverse genetics, BTV mutants that did not express NS4 protein but kept VP6 expression intact. These mutants replicated *in vitro* at a similar rate to the native viruses, showing that NS4 is dispensable for virus replication, at least in tissue culture (Ratinier et al., 2011). Additionally, there was no difference in the virulence of these mutants and the parental strains in two mouse models examined by the authors. The role of NS4 has not been elucidated, however, it might be involved in evasion of the host immune system. In BTV-8 the presence of intact NS4 allowed for more efficient replication in cells primed with

type I interferon (IFN), compared to NS4 deletion mutants (Ratinier et al., 2011). The role of NS4 in infection of the insect vector and the natural host has not been described to date. NS4 of BTV, and recently of AHSV, have been demonstrated to bind to dsDNA, however the significance of this finding has not been explored further (Belhouchet et al., 2011; Zwart et al., 2015).

1.6.8 Segment 10 ORF 2

Recent bioinformatics analysis of the BTV genome revealed the presence of yet another previously unidentified ORF (Sealfon et al., 2015). This approach identified an internal signal in S10 corresponding to a 50-59 codon-long ORF conserved among BTV isolates. Two initiation codons are present at the amino terminal of the sequence, the second of which is fully conserved while the first is absent in several isolates. Apart from the two AUGs and the stop codon, the rest of the sequence of this overlapping ORF is poorly conserved and majority of mismatches are non-synonymous (Sealfon et al., 2015). In addition, the second AUG sits in +1 frame of the late domain PPxY of the NS3 (Bhattacharya et al., 2015). This part of the sequence is does not allow much flexibility. It is unclear at present whether this ORF encodes a functional protein, and Sealfon and colleagues suggested the possibility that the sequence might encode an RNA structural element (Sealfon et al., 2015). A recent study demonstrated that a plasmid-expressed tagged product of this ORF localises in the nucleolus of transfected cells (Stewart et al., 2015). A luciferase expression assay showed that ORF 2 expressed protein inhibited gene expression, but not RNA translation. No differences were observed in the growth assays between wild type BTV and the mutant virus not expressing the ORF 2 protein. Moreover, the ORF 2 deletion mutants did not have any effect on BTV pathogenicity in a mouse model of disease. The authors suggested that the protein might play a role in infection of the *Culicoides* vector or the ruminant host (Stewart et al., 2015).

1.7 Clinical disease and pathogenesis

Hutcheon's first detailed description of bluetongue in sheep was published in 1881 where he referred to the disease as Epizootic Catarrh (Hutcheon, 1881). Animals affected by bluetongue initially display a fever that usually exceeds 40^{o}C and can last up to 7 days. The first characteristic signs of disease appear a few days later and include excessive salivation, facial oedema, nasal discharge and inflammation of the oral and nasal mucosa

(Cox, 1954; Elbers et al., 2009; Spreull, 1905). The onset of these symptoms might be preceded by 1-2 days of anorexia (Spreull, 1905). As disease progresses, erosions of the oral mucosa and characteristic lesions of the tongue and buccal mucosa become apparent and are often accompanied by swelling of the lips and tongue. At this stage, anorexia, apathy and depression are usually present (Elbers et al., 2008b; Erasmus, 1975). In severe cases, the tongue epithelium might become entirely necrotic, and then slough (Elbers et al., 2008c; Elbers et al., 2009; Hutcheon, 1881; Spreull, 1905). However, cyanosis of the tongue leading to a "blue tongue" is rarely seen in the field (Hutcheon, 1881). Respiratory distress is common and partially related to nasal congestion and encrustation of the nasal passage, and in severe cases caused by pulmonary oedema and pleural effusion (Cox, 1954). In the later stages of the disease, diarrhoea might occur, often containing mucus or blood (Cox, 1954; Erasmus, 1975).

Foot lesions are often present in sick animals. Generally, inflammation of the coronary band develops after nasal and mouth sores begin to heal. The extent of coronitis differs between affected sheep and may result in lameness and reluctance to stand. Sheep trying to walk and feed on their knees are a characteristic picture of BTV-induced coronitis (Erasmus, 1975).

Animals with severe bluetongue may show signs of muscle degeneration ranging from general weakness and prostration to sudden emaciation. Torticollis develops in some cases, usually around two weeks after disease onset (Cox, 1954; Spreull, 1905). Excessive desquamation and fleece shedding can occur (Elbers et al., 2008b; Erasmus, 1975). Mortality usually ranges from 2 to 30% but can reach much higher numbers in naïve susceptible flocks (Conraths et al., 2009; Cox, 1954; Erasmus, 1975; Szmaragd et al., 2007).

Until recently there has been little emphasis on bluetongue in cattle (Hourrigan and Klingsporn, 1975a). Although viraemic, infected cattle rarely develop symptomatic disease. The clinical presentation in animals that show signs of bluetongue is much milder than in sheep, and is rarely fatal. The most common clinical signs include fever, hyperaemia of the oral and nasal mucosae, mucopurulent nasal discharge, frothing, swelling of the tongue, ulceration and coronitis (Bekker et al., 1934; Dal Pozzo et al., 2009; Hutcheon, 1881; Thiry et al., 2006). Lactating cows can develop inflammation and

lesions of the teat and udder. Milk production is markedly reduced (Dal Pozzo et al., 2009; Thiry et al., 2006). Skin of affected animals might be inflamed, and necrotic lesions are often observed. Animals with acute dermatitis display photosensitivity and seek shaded areas at pasture (Bekker et al., 1934). Often, infected cattle show only one of the characteristic bluetongue signs combined with non-specific signs of infection such as apathy and inappetance (Williamson et al., 2008).

Other species of ruminants can also become infected with BTV. White-tailed deer are particularly susceptible to BTV and often succumb to severe and fatal disease (Thomas and Trainer, 1970). On the contrary, BTV-infected goats become viraemic but rarely display signs of disease (Caporale et al., 2014; Erasmus, 1975; Luedke and Anakwenze, 1972). In addition, antibodies against several BTV serotypes were detected in a range of carnivorous species including cheetahs, lions, hyenas and domestic cats and dogs in Africa (Coetzee et al., 2012a).

BTV invasion of a mammalian host begins when a carrier vector deposits infectious virions at the bite site. The initiation of infection is likely facilitated by insect-dependent factors that modulate the host's innate immune reaction (Pages et al., 2014). The virus then travels into regional lymph nodes where the primary replication takes place (Barratt-Boyes and MacLachlan, 1994; Barratt-Boyes et al., 1995; MacLachlan et al., 1990). Conventional dendritic cells in the lymph of the skin are specifically targeted by BTV and interestingly the virus infection prolongs their survival, making them an optimal vehicle for further spread (Hemati et al., 2009). From lymph nodes, BTV disseminates to a variety of tissues and virus replication takes place primarily in capillary endothelial cells, mononuclear cells and lymphocytes (Darpel et al., 2007; Maclachlan et al., 2009; Pini, 1976). Bovine monocytes can support BTV replication *in vitro*, however *in vivo* they are infected with low frequency and therefore unlikely to be a major source of viraemia in cattle (Whetter et al., 1989). BTV can also infect the γδ subset of T-lymphocytes without inducing shut-off of host protein synthesis or causing cell death (Takamatsu et al., 2003). In the bloodstream, a large fraction of BTV can be found associated with erythrocytes and thrombocytes, where it can persist for extended periods (Maclachlan, 1994). Indeed, BTV can be detected in the blood of infected animals for approximately 6 months, although infectious virus can only be isolated for up to two months post infection

(Barratt-Boyes and MacLachlan, 1994; Bonneau et al., 2002; Koumbati et al., 1999; MacLachlan, 2004).

Once vireamia is established (4-6 days post infection; d.p.i.) BTV can be detected in most organs, including the lymph nodes, spleen, lungs, heart and intestines (Pini, 1976). In the spleen, the majority of BTV-infected cells are found on the periphery of lymphoid follicles (MacLachlan et al., 1990). Lung tissue collected from sick animals often shows signs of oedema and inflammation with moderate infiltration of lymphocytes, plasma cells and eosinophils (Darpel et al., 2012). In the heart, BTV can be found associated with capillaries, but is not found in cardiac muscle cells. Similarly, infected capillaries can be detected in the lip, tongue and skin tissue (Darpel et al., 2012). Infection of the endothelium leads to increased vascular permeability and therefore is the major cause of activation of the inflammatory cascade and development of extensive oedema (Chiang et al., 2006).

Damage to endothelial cells and the subsequent inflammatory response are the likely mechanism leading to the pathology seen in infected animals. In particular, BTV infection was shown to induce expression of interleukin (IL) -1, IL-8 and vasoactive mediators *in vitro* in ovine (OvEC) and bovine endothelial cells (BoEC). Additionally, increased levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were found in BTV-infected BoEC. While COX-2 is considered an inflammatory mediator, it is also an important source of prostaglandins (prostacyclin in particular) and therefore can play a role in inhibition of platelet aggregation, indirectly reducing BTV-induced vascular damage (Cheng et al., 2002). A comparison between responses of lung endothelium of sheep and cattle to BTV challenge revealed that production of vasoactive mediators, and specifically the ratio between thromboxane and prostacyclin, was crucial in determining the degree of endothelial cell susceptibility to virus-induced damage (DeMaula et al., 2001; DeMaula et al., 2002a). Thromboxane is a vasoconstrictor and has prothrombic properties, while prostacyclin is a vasodilator and an inhibitor of platelet aggregation with anti-inflammatory functions (Cheng et al., 2002). Healthy endothelium maintains homeostasis between both types of prostaglandin. In cattle and sheep infected with BTV, the ratio of thromboxane to prostacyclin is increased indicating enhanced coagulation (DeMaula et al., 2002a). However, the increase of thromboxane is significantly greater in sheep (DeMaula et al., 2002a). Russell and colleagues compared responses of OvEC

and BoEC to BTV infection *in vitro* (Russell et al., 1996). The authors showed that infection of OvEC results in higher virus yields at 72 h.p.i., despite higher levels of IFN released by this cell line in response to infection (Russell et al., 1996). Overall, these observations could in part explain the factors behind the low incidence of symptomatic disease in infected and viraemic cattle in comparison to more severe bluetongue manifestations in sheep (Maclachlan et al., 2009).

1.8 Epidemiology

1.8.1 Overview of global BTV distribution

There are currently 27 serotypes of BTV recognised and distributed across all continents with the exception of Antarctica (Jenckel et al., 2015; Maclachlan, 2011). The global distribution of specific strains/serotypes is constantly changing and new incursions happen frequently. In recent years, BTV distribution has been expanding northwards and now includes areas between latitudes of 34°S and 53°N. Two geographic groups of BTV have been described based on phylogenetic studies and they divide the strains into western and eastern "topotypes" that include the Americas and Africa, or the Middle East, Asia and Australia, respectively (Bonneau and MacLachlan, 2004). Figure 4 shows the serotypes reported in specific regions of the world.

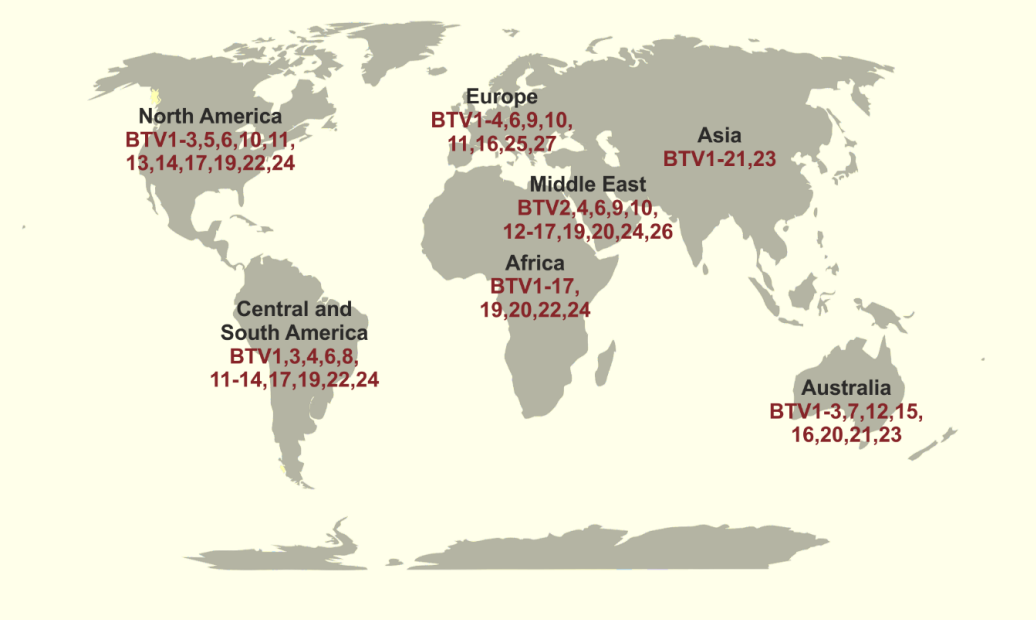

Figure 4. Global distribution of BTV. Based on data obtained from: http://www.reoviridae.org/dsrna_virus_proteins/ btv-serotype-distribution.htm, edited by Mertens *et al.* (accessed June 2015)

Prior to 1998, BTV incursions into Europe occurred very infrequently and affected only countries of the Mediterranean Basin including Spain, Portugal, Cyprus and Greece (Mellor and Wittmann, 2002). The situation changed dramatically when a BTV-9 outbreak occurred on several Greek islands and spread northwards and westwards affecting nine other countries. Further incursions of BTV-1, -4 and -16 from Greece and separately from Morocco followed shortly after. With the additional introduction of BTV-2 into Italy from Northern Africa, it became clear that the environment of Southern Europe was sufficient to support the spread of BTV (Purse et al., 2005). In 2006, BTV-8 was first introduced to the Netherlands by unknown means and expanded across Europe, reaching as far north as Scandinavia. Shortly after, BTV-1 emerged in Southern Europe and by 2008, it was detected in several countries including Spain, Portugal and France (Wilson and Mellor, 2009). In the same year, an additional serotype, BTV-6, was reported in the Netherlands and in Germany, and it appeared to be related to the modified live vaccine strains from South Africa (Eschbaumer et al., 2010; Maan et al., 2010). Moreover, surveillance in Switzerland detected a novel virus that caused disease in goats and did not belong to any known BTV serogroup. The virus was first called Toggenburg and later, based on sequence and phylogenetic analysis, reclassified as BTV-25 (Hofmann et al., 2008). Another novel serotype, BTV-27, was reported in 2014 in the Corsica region of France. The virus was closely related to BTV-25 from Switzerland and BTV-26 from Kuwait (Jenckel et al., 2015).

The reasons behind the sudden spread of BTV in Europe are still a matter of debate although several factors, including increased vector range, climate change (especially milder temperatures in winter) and effective overwintering mechanisms are likely to play a crucial role in the process (Purse et al., 2005). Moreover, the emergence of novel serotypes in distinct areas of Europe could reflect a rapid adaptation of BTV to new environments and the available vector and host species.

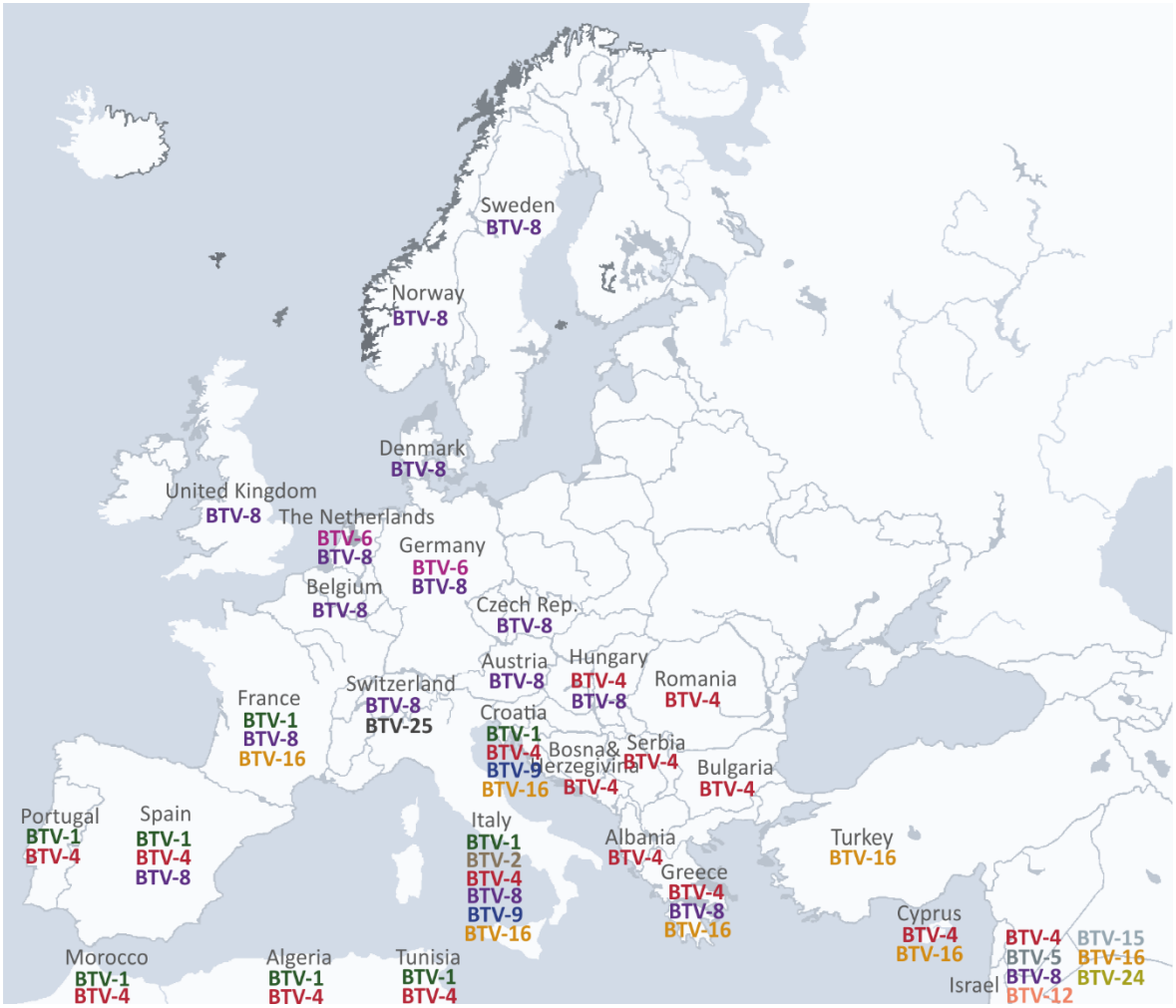

Figure 5. BTV serotype distribution in Europe and the Mediterranean Basin in 2004-2014. Representation of BTV distribution based on data obtained from OIE-BT-Labnet *http://oiebtnet.izs.it/oiemaps/*

1.8.2 Vector and transmission

BTV is transmitted from an infected host to a susceptible animal primarily by biting midges, *Culicoides spp*. The earliest concept of bluetongue as an insect transmitted disease came from observations of disease occurrence patterns in South Africa. In 1905, Spreull noted that bluetongue was most common in warm seasons, and depended on rainfall, and outbreaks cease after the first frost season. Additionally, flocks grazing at high altitudes and kept indoors overnight did not contract BTV, and animals with unshorn wool were more resistant to infection (Spreull, 1905). In 1944, du Toit experimentally infected sheep with homogenates of either wild-caught *Culicoides* midges or midges fed on infected animals. Consequently, the sheep developed bluetongue, which confirmed that *Culicoides* was a vector of bluetongue (du Toit, 1944). The virus has also been shown

to cross the placental barrier leading to foetal infection, the phenomenon attributed mainly to egg and cell passaged strains (Parsonson, 1990). Experimental infection of IFNAR$^{-/-}$ mice showed that BTV-8 could be spread orally between infected and naïve animals (Calvo-Pinilla et al., 2010). Recently, Batten and colleagues showed that BTV-26 was not able to replicate in *C. sonorensis* but it was spread in goats via direct contact (Batten et al., 2014). Additionally, there is evidence that suggests that BTV can spread via an oral route to certain African carnivores, but their role in the natural infection cycle is unclear (Alexander et al., 1994).

Culicoides biting midges are extremely common worldwide and although over 1000 species are recognised, only a few have been shown to be able to transmit BTV efficiently (Meiswinkel et al., 2004). Competent vector species differ between continents. *C. imicola* is the traditional BTV vector commonly found in Africa and parts of Asia, as well as Southern Europe (du Toit, 1944; Maclachlan, 2010b, 2011; Mellor et al., 1984; Savini et al., 2005). This species, however, is not present in the north of Europe and the transmission of BTV-8 was therefore attributed to Palearctic species of *Culicodes* including *C. obsoletus*, *C. pulicaris*, *C. dewulfi* and *C. chipterus* (Caracappa et al., 2003; Purse et al., 2004; Savini et al., 2004). It has been suggested that certain species of *Culicoides* might be competent vectors for only specific serotypes of BTV (Tabachnick, 2004). In particular, *C. sonorensis* has been associated with BTV- 10, 11, 13 and 17 spread in Northern America, while *C. insignis* is a vector of BTV-1, 3, 6, 8, 12 and 14 in South America (Lager, 2004; Tabachnick, 2004). BTV-2 has been isolated only in southern parts of the USA likely due to specific association with *C. insignis* that is not found in the northern parts of USA (Gibbs and Greiner, 1994; Maclachlan, 2011). This division between the occurrence of a specific competent vector in a confined geographic area and the prevalence of distinct BTV serotype could involve factors such as vector-virus interactions, availability of a particular host species or other environmental factors (Tabachnick, 2004).

C. sonorensis is one of the common vector species of BTV. The rate of transmission by this species is extremely high and a single bite of an infected midge is sufficient for infection of the natural host (Baylis et al., 2008). Transmission of BTV from an infected animal to a vector occurs at a much lower frequency (estimated transmission at less than 1% efficiency). Although in experimental conditions 70% of midges feeding on viraemic

sheep had detectable BTV titres immediately after a blood meal, only 0.6% became infected with BTV (Baylis et al., 2008). This suggests the presence of barriers within the vector that constrain viral growth or dissemination to salivary glands where BTV replicates prior to release into the mammalian host during the bite of an infected midge (Fu et al., 1999). Three of such barriers have been identified in *C. variipennis*. The mesenteron infection barrier prevents establishment of infection in the midgut. The mesenteron escape barrier limits the replication to the cells of the gut and this dissemination barrier can impede the spread of BTV from the haemocoel to the secondary sites of replication (Fu et al., 1999). These barriers might effectively stop the transmission of BTV in the majority of *C. variipennis*, referred to as refractory midges. The remaining susceptible population is transmission competent and serves as a genuine vehicle that allows BTV spread in the natural infection cycle (Fu et al., 1999; Mellor, 1990). The traits that govern susceptibility of an individual midge to bluetongue infection are genetic, and cross-breeding of the midge population can lead to selection of insects that are either highly susceptible or refractory to BTV (Jones and Foster, 1974; Mellor, 1990). Tabachnick identified a single genetic locus, *blu* that controlled *C. variipennis* competence for BTV infection (Tabachnick, 1991). Interestingly, the rate of transmission by *Culicoides* is extremely high and a single bite from an infected midge might be sufficient for infection of the natural host (Baylis et al., 2008). Viraemia and antibody levels increase, however, with the number of infected midges that feed on the host, and the BTV titre of the vector (Baylis et al., 2008).

Transplacental transmission of BTV had been associated mainly with the use of live vaccine strains passaged multiple times in embryonated chicken eggs or in tissue culture. In 1955, Schultz reported that a significant number of lambs were aborted or born with severe developmental abnormalities after a vaccination campaign in California. Cerebral malformations were most common, often leading to so called “dummy lamb syndrome”, where the animals would be unresponsive to sensory stimuli, would not feed and would often walk in circles (Schultz and Delay, 1955). Sheep vaccinated between 30-80 days of gestation with live attenuated strains of BTV are most likely to give birth to congenitally deformed lambs, but the degree of pathology differs depending on the period of gestation. Specifically, experimental infection with the vaccine strain on the 40th day of gestation resulted in hydranencephaly and development of subcortical cysts in 20% of

lambs, while infection on the 75th day caused mild, and predominantly focal, lesions and porencephaly at birth. BTV does not naturally infect mature cells of neural origin, instead it replicates in precursor cells (neuronal and glial) found in the subependymal plate prior to their migration to the cerebral cortex. The virus then induces lysis of the undifferentiated cells leading to widespread necrosis and hydranencephaly (Osburn, 1994; Osburn et al., 1971a; Osburn et al., 1971b). At later stages of gestation, the only susceptible cells are undifferentiated glial cells and these are selectively targeted by BTV. In cattle, the highest susceptibility of foetuses to BTV-induced malformations occurs between 70-85 days of gestation (Waldvogel et al., 1992).

Transplacental spread of BTV seems to be primarily associated with the culture-adapted strains. This could be limited by controlled timing of vaccination programmes so that pregnant animals were not inoculated during the susceptible period of gestation. However, when the novel BTV-8 strain emerged in Northern Europe, it was reported to cross the placenta in more than 10% of cases. Since the virus was responsible for high mortality and morbidity of hundreds of thousands of animals, the fact that it was also transmissible from the mother to offspring became a new concern, not only due to potential reproductive losses but also as a possible cause of viral persistence in the population, and overwintering (Gibbs et al., 1979; Wilson et al., 2008). However, recent studies comparing vertical transmission of minimally passaged BTV-8 and BTV-2 in experimentally inoculated ewes showed higher frequency of transplacental spread in sheep infected with the latter serotype (Rasmussen et al., 2013). It is therefore possible that other serotypes/strains of BTV are equally capable of crossing the placenta, at least in experimental situations. Molecular determinants enabling BTV to cross the placenta have not been described to date.

1.9 Mouse models of bluetongue infection

Neonatal mice have been used as a model to study the virulence of BTV strains and specific aspects of bluetongue pathogenesis (Caporale et al., 2011; Carr et al., 1994; Franchi et al., 2008; Waldvogel et al., 1987; Waldvogel et al., 1986). Mouse death is caused invariably by necrotising encephalitis and mortality is affected by the route of virus inoculation (Waldvogel et al., 1987). Subcutaneous injection has been used in studies to compare neuroinvasiveness of BTV strains, whereas mortality of newborn

mice inoculated intracranially has been shown to be an indicator of attenuation of vaccine strains (Brewer and Osburn, 1998; Carr et al., 1994; Franchi et al., 2008; Waldvogel et al., 1986). Neonatal mice are useful in differentiating between virulent and avirulent BTV strains, however they do not permit the in-depth study of the mechanisms of infection, nor reflect the pathology associated with virus dissemination within the host. Moreover, an adult model is necessary to identify host factors involved in adaptive immunity to BTV.

Calvo-Pinilla recently developed a new small animal model of bluetongue (Calvo-Pinilla et al., 2009a). Adult IFNAR$^{-/-}$ mice on a C57BL/6 genetic background deficient in interferon alpha and beta receptors are susceptible to infection with different BTV serotypes, and display visible signs of infection such as ocular discharge, fur ruffling and apathy. IFNAR$^{-/-}$ mice have been used to compare virulence of different BTV strains, as the animal mortality rate gives an indication of the degree of BTV serotype/strain pathogenicity (Calvo-Pinilla et al., 2009a; Ratinier et al., 2011). Dissemination of BTV in the IFNAR$^{-/-}$ mice resembles infection in the natural host. After intravenous inoculation, high levels of BTV are first detected in the spleen followed by an increase in BTV levels in the blood and virus dissemination to other tissues including lymph nodes, lungs and thymus. Similar to the natural host, IFNAR$^{-/-}$ mice infected with BTV show widespread oedema. Histopathological examination of tissues collected from infected animals show microscopic lesions present in the lungs and spleen. Lymphoid depletion and neutrophil infiltrates are observed in the white pulp of the spleen and the lungs show signs of interstitial pneumonia (Calvo-Pinilla et al., 2010; Calvo-Pinilla et al., 2009a). Vaccinated IFNAR$^{-/-}$mice develop neutralizing antibodies and are protected against lethal challenge with virulent BTV strains, which makes them a good model for evaluating efficacy of modified live vaccines (MLV) against BTV (Calvo-Pinilla et al., 2012; Calvo-Pinilla et al., 2009b).

Combining experiments using IFNAR$^{-/-}$ mice and neonatal mice in BTV research can provide insight into different aspects of infection. Indeed, two recent studies took advantage of both BTV models to assess pathogenicity of BTV *in vivo* (Caporale et al., 2011; Ratinier et al., 2011). Caporale and colleagues showed that a minimally passaged strain of BTV-9 that caused over 95% mortality in 3-day old NIH-Swiss mice was avirulent in the IFNAR$^{-/-}$ model (Caporale et al., 2011). Comparison of survival rates in both models

can allow more precise definition of the degree of virus attenuation, which is especially important in the development of live vaccines.

1.10 Host factors influencing outcome of bluetongue infection

Although BTV can infect all species of domestic ruminants, severe clinical manifestations are only commonly found in sheep (described in section 1.7). Sheep inoculated with blood from viraemic animals generally develop acute infection with overt symptoms of bluetongue while cattle show only mild signs of infection (Bekker et al., 1934; Spreull, 1905). The difference in morbidity and mortality of cattle and sheep infected with BTV has been attributed to the difference in severity of microvascular lesions in these animals (as described in chapter 1.7) (Russell et al., 1996). Goats inoculated with infectious blood remain asymptomatic, even when infected with blood passaged multiple times in the goat (Bekker et al., 1934; Cox, 1954; Erasmus, 1975; Spreull, 1905)

Early studies that compared the severity of bluetongue in sheep of diverse breeds in field and in experimental conditions found that there are marketed differences, particularly between indigenous African and imported European breeds (Spreull, 1905). Dorset Horn and Dorset Poll are generally considered highly susceptible to BTV (Jeggo et al., 1987). Merino, Sardinian and East Friesian sheep were reported to suffer from more severe symptoms and more frequently succumb to infection than African or Asian breeds such as Awassi and Damani (Cox, 1954; Sellers, 1984). However, outbreaks of symptomatic bluetongue were also reported in local breeds in Sudan and Iraq (Sellers, 1984). In recent work, no significant differences were seen between Swiss sheep and Dorset Poll (Worwa et al., 2008) or between mixed, Sardinian or Dorset Poll breeds (Caporale et al., 2014). Cattle of most breeds, including Friesland, Afrikander, Hereford and Shorthorn, were reported to be equally susceptible to BTV and to rarely develop severe disease (Bekker et al., 1934; Hourrigan and Klingsporn, 1975a).

It is important to stress that there are marked differences in response to BTV between animals of the same breed due to their genetic makeup and immunological status. For example, a “Report of the Cattle and Sheep Diseases for 1876” stated that “fat” sheep were more susceptible to bluetongue (Cox, 1954). Extensive immunological studies performed over several years by Neitz showed that individual Merino sheep injected

with the same infectious blood inoculum develop a wide range of symptoms, from mild transient fever to full-blown, fatal disease (Neitz, 1948).

Understandably, previous exposure to BTV and presence of neutralising antibodies have major influence on morbidity and mortality caused by bluetongue and it is one of the factors that explains why major outbreaks occur when naïve sheep are imported to BTV endemic regions (e.g. introduction of Merino to South Africa) or when the virus emerges in a new area for the first time (Jeggo et al., 1983; Schwartz-Cornil et al., 2008). In addition, co-infection with another pathogen can suppress or sensitise an animal's immunity to such an extent that it becomes more susceptible to BTV infection or displays a severe inflammatory reaction (Howell et al., 1970). Treatment of calves with dexamethasone prior to inoculation with BTV was shown to facilitate the development of severe symptoms in experimental conditions, which shows that innate immunity is also in part responsible for the host's resistance to bluetongue (Hourrigan and Klingsporn, 1975a).

Age has also been quoted as a factor determining the clinical outcome of BTV infection. According to Neitz, lambs of immunologically naïve mothers are susceptible to infection, but they tend to present milder symptoms than those seen in older animals (Neitz, 1948). Bekker reported that bluetongue rarely occurred in young calves. The apparent "resistance" of young animals could be explained by either passive immunity in suckling animals, or by the fact that calves tend to be kept indoors, thus decreasing the chances of being exposed to biting midges (Bekker et al., 1934). On the other hand, Spreull reported that young lambs, especially those in poor physical condition, succumbed to bluetongue more easily than adults and more often suffered from a gastrointestinal presentation followed by death (Spreull, 1905). In recent work, Caporale *et al.* showed that under standardised experimental conditions, 8-month old Dorset Poll sheep inoculated with BTV-8 showed no statistical difference in total clinical score compared with 2-year old animals of the same breed (Caporale et al., 2014).

1.11 Determinants of viral virulence

In addition to host-related factors, differences in clinical outcomes of BTV infection have been associated with specific serotypes or strains of the virus itself (Caporale et al., 2014; Maclachlan et al., 2009; Sellers, 1984). To date, 27 BTV serotypes have been reported,

generating variable levels of cross-protection and varying disease severity. In Europe, at least 11 different serotypes circulated over the years, but attention has been brought to BTV-8, in part due to its particularly high virulence. The differences between the pathogenicity of specific serotypes and strains, however, were previously noted in early studies of BTV in the natural host.

Neitz reported up to 72% mortality in sheep inoculated with blood containing serotype 4 ("Bekker" strain) while infection with serotype 8 ("Camp" strain) was generally non-lethal. No differences in virulence were noted between two strains of serotype 3 ("Mimosa Park" and "Cyprus") (Neitz, 1948). Interestingly, the severity of the clinical signs or pyrexia does not necessary correlate with mortality in sheep. Howell *et al.* observed that sheep inoculated with clinical isolates of BTV-3 displayed a range of symptoms (from mild to moderate) but all eventually succumbed to disease (Howell et al., 1970). However, animals infected with BTV-5 showed invariably severe bluetongue expression but recovered from the infection. Similarly, variation in clinical presentation was reported in sheep experimentally infected with BTV-16, BTV-10 and BTV-4, the last inducing the highest mortality (Goldsmit et al., 1975). Hooper and colleagues compared the virulence of South African (RSA) and Australian strains of BTV in groups of five Merino sheep and found that overall, RSA viruses were more pathogenic than homologous serotypes from Australia (Hooper et al., 1996). The RSA strain of BTV-3 caused particularly severe disease that culminated in the death of four sheep. Interestingly, of the four RSA serotypes tested (BTV-1, -2, -3, -4), only BTV-4 caused mild symptoms, which were comparable to Australian strains (Hooper et al., 1996).

Comparison of clinical signs associated with different BTV serotypes that circulated in Israel from year 2008 to 2011 showed that during these years, cattle on different farms suffered from a variety of 'clinical syndromes' that were associated with one specific BTV serotype (Brenner et al., 2011). BTV-8 exhibited relatively low virulence in animals in Israel but BTV-8 introduced to Northern Europe in 2006 caused severe disease and major losses of affected sheep. Similar observations were made with BTV-24, which was innocuous in the United States, yet in Israel was responsible for high morbidity in affected flocks (Brenner et al., 2010; Maclachlan, 2010a). Using an Italian strain of BTV-2 and Northern European BTV-8, both passaged once in KC cells and twice in BHK-21 cells, Caporale *et al.* demonstrated a similar degree of virulence of these two serotypes.

Interestingly however, the authors noted that there was a significant difference between two different strains of BTV-8 from Europe, with the Italian strain (isolated in 2008) being highly attenuated compared with the strain isolated in the Netherlands (Caporale et al., 2014).

In light of these data, it is clear that serotype by itself cannot be used as a reliable indication of the pathogenicity of a specific BTV isolate. In addition, only VP2 is the determinant of BTV serotype, other proteins may contribute to the pathogenicity of specific strains. Through natural reassortment of genomic segments within one host or vector, BTV can acquire new pathogenicity traits, yet remain within the same serotype group. With multiple serotypes and strains of BTV circulating in one area the possibility of reassortment is also quite high (Batten et al., 2008b; Maan et al., 2015; Nomikou et al., 2015; Oberst et al., 1987; Sugiyama et al., 1982).

The study of determinants of BTV pathogenicity had long been hampered by the unavailability of a small animal model to replace the complex system of a natural host. Therefore, the majority of data regarding BTV pathogenicity have been obtained by field observations rather than laboratory experiments. The establishment of the IFNAR$^{-/-}$ mouse model (2009) that reproduces many aspects of bluetongue infection in ruminants was a major step that facilitated the study of the molecular determinants of BTV pathogenesis (Calvo-Pinilla et al., 2009a). Caporale *et al.* used IFNAR$^{-/-}$ mice to examine differences in virulence of viruses that have been maintained at low or high passage *in vitro* (Caporale et al., 2011). In this study, attenuated strains of BTV-2, -4, -9, showed amino acid changes occurring consistently in VP1, VP2 and NS2, when compared to the related virulent strains. The authors speculated that these proteins contained major determinants of pathogenicity. They did not exclude the possibility that attenuation could have been a result of a specific combination of mutations occurring within the 10 segments of the BTV genome.

VP2 has been previously suggested to be one of the virulence factors in the Australian strain of BTV-1 (Gould and Eaton, 1990) and BTV-17 isolated in the USA (Bernard et al., 1994). Comparison of nucleotide sequences between pathogenic BTV-1AUS and an non-pathogenic strain with multiple *in vitro* passage history revealed presence of ten nucleotide mismatches, four of which lead to amino acid changes (Gould and Eaton,

1990). The non-synonymous mutations likely contributed to attenuation of the virus in the natural host. Unfortunately, the remaining genomic segments were not sequenced and therefore not all putative determinants of pathogenicity of BTV-1AUS were described. In order to recreate the results of this study in an independent experiment, the virulent strain was passaged in chicken embryos and multiple times in tissue culture and the resulting strain was used to inoculate sheep (Gould and Eaton, 1990). Again, the tissue culture adapted strain was attenuated *in vivo* but no differences in nucleotide sequence of VP2 were found when compared with the parental virus. The authors hypothesised that in the absence of amino acid changes in VP2, mutations in other proteins, such as VP5 and VP7, could have changed conformation in the outer capsid resulting in altered tropism or virulence (Gould et al., 1988).

Carr and colleagues studied factors involved in neurovirulence of BTV-11 using an attenuated strain UC-2 and a virulent strain UC-8 (Carr et al., 1994). In adult ruminants, BTV does not normally possess neurotrophic properties but it can cause malformations of the brain of the developing foetus (Richardson et al., 1985). Similarly, most wild-type strains of BTV are pathogenic in newborn mice and when injected intracerebrally, they cause extensive necrotising encephalitis (Caporale et al., 2011; Carr et al., 1995; van der Sluijs et al., 2013). Carr *et al.* generated reassortant viruses between neurovirulent UC-8 and the non-pathogenic UC-2 and assessed their virulence in subcutaneously inoculated newborn BALB/c mice (Carr et al., 1994). Viruses that contained segments 4, 8, 9 and 10 of UC-2 were partially attenuated and the viruses containing either segments 4, 5 and 10 or 1, 3, 4, 5, and 6 caused equal or lower mortality than UC-2. Based on these observations, the authors concluded that S5 (corresponding to VP5 in that study) was likely the main determinant of BTV neuroinvasiveness (Carr et al., 1994). It is possible however, that other proteins, such as VP4, also contributed to UC-2 attenuation, but this option has not been discussed further.

A few studies attempted to identify the determinants of pathogenicity of BTV or AHSV by generating reassortants between two parental viruses of different serotypes (Celma et al., 2014; O'Hara et al., 1998). O'Hara and colleagues assessed virulence of reassortants between the non-pathogenic strain of AHSV-8 and a pathogenic strain of AHSV-3 in Balb/c mice (O'Hara et al., 1998). They found that replacement of AHSV-3 S2 with the equivalent in AHSV-8 was sufficient to attenuate the virus completely. Minor

reduction in virulence was found in the reassortant that contained AHSV-8 VP1 in the AHSV-3 backbone (80% mortality). Several other reassortants displayed a 'novel' phenotype characterised by intermediate mortality and morbidity (ranging from 20% - 80%) compared with parental viruses. The authors concluded that the intermediate pathogenicity in the mouse model was conferred by the presence of S2, S5 and S6 (coding for VP2, VP5 and NS1 respectively) from AHSV-8 and S10 (NS3) from AHSV-3. AHSV-3 NS3 alone was not sufficient to confer any degree of virulence. However, reassortants containing AHSV-3 NS3 in conjunction with AHSV-3 VP7 and VP1 and the remaining proteins from AHSV-8, caused mortality and morbidity comparable with the virulent ASHV-3. Moreover, reassortant A79 (AHSV-8 backbone + AHSV-3 S7 and 9) produced very small plaques *in vitro* while A790 (AHSV-8 backbone + AHSV-3 S7, S9 and S10) produced normal size plaques. Based on their data, the authors suggested that molecular interactions between serotype specific forms of VP2, VP5 and NS3 (and possibly other structural proteins) played a major role in determining the phenotype of AHSV reassortants in mice (O'Hara et al., 1998). The reassortment of diverse serotypes of AHSV or BTV in the field (including vaccine strains) could therefore lead to emergence of viruses with superior pathogenicity.

A recent study examined a role of serotype specific VP2, VP5 and NS3 in the pathogenicity of BTV by assessing the virulence of BTV-1/BTV-8 reassortants with exchanged S2, S6 and S10 in sheep (Celma et al., 2014). First, the authors performed titrations of parental and various reassortants in ovine SFT-R cells. BTV-1 replicated more efficiently than BTV-8 in this cell line. However, BTV1/8NS3 produced yields comparable to BTV-1 while BTV1/8VP5.NS3 replicated least efficiently. Clinical scores of animals infected with the various reassortants showed no significant differences compared with parental virus-infected sheep. The authors argued however, that of all tested viruses, BTV1/8VP2.5.NS3 produced most of the different clinical manifestations that did not resemble parental BTV-1 phenotype (Celma et al., 2014). It is difficult to interpret the *in vivo* data, especially in light of the fact that little variation in virulence was observed between the parental viruses. The differences in replication kinetics of the reassortants used in this study could again point at the multigenic nature of BTV fitness *in vitro* and the more complex mechanisms of pathogenicity in the natural host.

Coetzee and colleagues tried to determine if BTV phenotype *in vitro* was a correlate of pathogenicity *in vivo* (Coetzee et al., 2014). Replication kinetics, cell viability and apoptosis/cytotoxicity induced by two rescued avirulent strains of BTV (BTV-1 and BTV-6, rgP1 and rgP6, respectively), virulent strain of BTV-8 (rgP8), and various reassortants between these three parental viruses, were assessed in Vero cells (Coetzee et al., 2014). In general, rgP1 produced the highest yields from all parental viruses, which showed that the ability of a strain to replicate efficiently *in vitro* did not necessary correlate to its virulence *in vivo*. A similar conclusion was reached for the other two parameters as no difference was observed between cell viability and cytotoxicity induced by rgP6 and rgP8. One reassortant virus that contained the backbone of rgP1 and segments 2, 6, 7 and 10 (VP2, VP5, VP7 and NS3 respectively) of rgP6 had a very different phenotype compared to the parental viruses. Its replication kinetics resembled that of rgP6 yet it caused very little apoptosis/cytotoxicity, and the cells infected by this strain remained the most viable of all tested samples 72 h.p.i. For other reassortants, having S5 and S8 of rgP8 correlated with decreased cell viability compared to parental viruses, while having S10 of rgP8 in the rgP6 backbone reduced the reassortant ability to cause apoptosis (Coetzee et al., 2014). The data demonstrated that the complete BTV phenotype is governed by gene constellations and while specific genes might confer individual characteristics, combinations of proteins could equally contribute to the virus fitness and virulence (Coetzee et al., 2014).

Altogether, these studies show that several factors, both host and virus related, contribute to the overall clinical presentation and the outcome of bluetongue in the mammalian host. Additionally, environmental stress, such as UV irradiation, can trigger a more severe response to infection. In light of the involvement of so many variables, it is difficult to weight the influence of individual elements confidently. Moreover, the majority of work describing factors that determine the clinical outcomes of BTV infection come from early, poorly controlled studies or from field observations and therefore have to be interpreted with caution.

1.12 BTV interactions with host interferon system

It has been known for a long time that BTV is a strong inducer of interferon (IFN) *in vitro* and *in vivo* (Doceul et al., 2014; Foster et al., 1991; Huismans, 1969; Jameson et al., 1978;

Vitour et al., 2014). However, in recent years there has been increasing interest in exploring the role of type 1 IFN in BTV infection and the viral means to counteract IFN-related immune responses.

Ruminants at different stages of development are able to produce IFN in response to BTV infection (Maclachlan et al., 1984; Maclachlan and Thompson, 1985). Experimental infection of bovine foetuses at 125 days of gestation with BTV-10 resulted in IFN production in tissues and in the serum, which might have played a role in limiting the dissemination of the virus (Maclachlan et al., 1984). Calves inoculated intravenously with BTV-10 produced high levels of IFN soon after infection, which persisted for a maximum of 4 days. Hence, MacLachlan and colleagues concluded that the IFN response was important primarily for controlling the initial stages of BTV infection (Maclachlan and Thompson, 1985). In sheep infected with various serotypes of BTV, IFN was detected in serum at 5 d.p.i. and reached the highest levels at 6 d.p.i (Foster et al., 1991). At the same time, a first peak of viraemia was observed. Interestingly, the levels of IFN decreased at day 9 after infection, which correlated with the second increase in BTV titres in the blood that occurred a day later. The authors speculated that the biphasic nature of the viraemia could have been related to induction of the IFN response followed by adaptive immune responses (Foster et al., 1991). Increased levels of type I IFN were also observed in skin lymph of sheep inoculated intradermally with BTV-2 and BTV-8, with the peak levels of IFN detected at 5-6 d.p.i (Ruscanu et al., 2012).

BTV has been shown to be an extremely potent IFN inducer in mice (Jameson et al., 1978). Infection with $10^{7.5}$ PFU of a vaccine strain of BTV induced up to 250,000 IFN U/ml in mouse sera, which was over 10 times higher than any other agents used in the study (including Reovirus 3, Newcastle disease virus, endotoxin etc.). The levels of IFN peaked at 8 h.p.i. and declined rapidly afterwards. Interestingly, high levels of IFN were also detected when UV-inactivated virus was used as inoculum.

Knocking-out the IFNAR receptor makes mice susceptible to lethal infection with BTV (Calvo-Pinilla et al., 2009a), which suggests that extreme levels of IFN prevent successful infection in this species. Moreover, the difference between the strength of the IFN response in ruminants and rodents could be potentially explained by host-specific IFN antagonism by one of BTV's molecular factors (Parisien et al., 2002; Webby et al., 2004).

Different cell lines express variable amounts of IFN in response to BTV infection, which might be due to their intrinsic properties, or to the ability of BTV to counteract IFN production in particular cell types (Chauveau et al., 2012; Doceul et al., 2014; Jameson and Grossberg, 1979; Ruscanu et al., 2012; Russell et al., 1996). For example, BTV-8 infection of bovine endothelial cells caused >2000 fold increase in IFN-β mRNA expression while more than a 10,000 fold increase was observed in A549 cells (human pulmonary adenocarcinoma cells) (Chauveau et al., 2012). Interestingly, a difference between IFN-β expression in response to infection with diverse serotypes has been described (Chauveau et al., 2012). Similarly, Fulton and Pearson observed differences in the amount of IFN produced in bovine and feline cell lines in response to BTV -10, -11, -13, and -17 (Fulton and Pearson, 1982). These data suggest that individual serotypes/strains of BTV might not be equally capable of modulating the IFN response in the infected cells. Multiple passage *in vitro* in cells with a defective IFN system might result in the generation of viruses with decreased capability to counteract IFN-related host responses and therefore the passage history should be considered when assessing the IFN response to a specific BTV strain (Janowicz et al., 2015; Perez-Cidoncha et al., 2014).

In non-hematopoietic cells, BTV is sensed primarily by RIG-I and Mda5 and silencing of either of the helicases or one of the adaptor proteins MAVS leads to a significant decrease in type I IFN produced in response to infection (Chauveau et al., 2012). Moreover, IFN expression is not induced by UV treatment of BTV, which shows that replication is essential for virus sensing and activation of the IFN cascade in non-hematopoietic cells. On the contrary, in plasmacytoid dendritic cells (pDC), type I IFN is also upregulated due to stimulation with UV-inactivated BTV. In pDC infected with BTV, the IFN cascade is triggered through the MyD88 adaptor and is independent of TLR7/8 activation (Ruscanu et al., 2012). The primary sensor of BTV in pDC has not been described to date but Ruscanu *et al.* speculated that some novel helicases coupled with MyD88 might be involved in sensing of BTV dsRNA in the cytosol (Ruscanu et al., 2012).

BTV, like other viruses, has evolved several strategies to counteract the host IFN response. Specifically, BTV has been shown to interfere with STAT-1 phosphorylation and translocation to the nucleus (Doceul et al., 2014). Reduced STAT-1 phosphorylation *in vitro* was not observed in cells infected with UV treated BTV and therefore replication,

or protein expression, seems to be crucial for inhibition to occur. Moreover, the level of phosphorylation in Vero cells (infected with 0.1 $TCID_{50}$/cell) was not affected at an early time point after infection (3 and 6 hours) while it was prominent at 24 h.p.i. and complete at 42 h.p.i. BTV-8 and BTV-4 equally inhibited STAT-1 phosphorylation suggesting that the process was not serotype/strain specific. At 24 h.p.i. levels of JAK, TYK, STAT-2 or their phosphorylation were not affected by BTV infection. However, at 42 h.p.i. their expression in A549 and Vero cells was markedly reduced (Doceul et al., 2014). It is possible therefore that BTV actively inhibits phosphorylation of STAT-1 while the down-regulation of the other proteins at later time points might be caused by generalised virus-induced protein translation shutdown (Mertens et al., 1984; Ratinier et al., 2011).

To date, only two BTV proteins have been implicated in interactions with the IFN system (Chauveau et al., 2013; Doceul et al., 2014; Ratinier et al., 2011). In a recent study, the effect of products expressed from different BTV ORFs on the activation of the IFN-β promoter in 293T cells was assessed (Chauveau et al., 2013). Among various BTV proteins, NS3 was shown to reduce IFN-β promoter activation most efficiently. This effect was conserved between wild type and vaccine strains of BTV-8, BTV-4 and BTV-2, although NS3 of vacBTV-2 was less potent than then the other ORFs tested. Neither C-Jun-driven transcription nor CMV promoter activation were inhibited by NS3, suggesting that the effect is IFN-β promoter specific. NS3 was demonstrated to have an effect on transcription of IFN-β gene expression and consequently reduced expression of several interferon stimulated genes (ISG). However, the exact mechanism of NS3 activity was not determined although it was narrowed down to pathways acting downstream of RIG-I and upstream of TBK1/IKKε.

NS4 is the second of the BTV proteins suggested to interact with the host cell innate immune system (Ratinier et al., 2011). In cells pre-treated with universal IFN, NS4 confers a replication advantage to BTV serotype 8. However, it seems to be dispensable for serotype 1 under similar conditions. Ratinier and colleagues speculated that since NS4 has a leucine zipper motif it could bind nucleic acids and control the transcription of genes involved in the induction of the IFN cascade (Ratinier et al., 2011). More studies to describe the role of NS4 in BTV interactions with the host are currently ongoing.

The antiviral IFN response is an essential tool to control BTV replication in the mammalian host. In the insect vector however, the IFN system does not exist and a different mechanism is utilised to limit dissemination of the virus. Early reports showed that *Culicoides* cells (KC) could be persistently infected with BTV without causing cytopathic effects. Interestingly, at late stages after infection (>14 d.p.i.) only single large VIBs, likely formed by fusion of individual smaller bodies, was present in the cytoplasm of infected cells. The cells containing these giant VIBs, however, contained very few BTV particles, suggesting that insect cells were able to inhibit viral replication (Fu, 1995). Deep sequencing of KC cells infected with BTV revealed the presence of 21-nucleotide long virus-derived small interfering RNAs (viRNAs) directed against eight of the BTV genomic segments (Schnettler et al., 2013). This demonstrated that vector cells mount an iRNA response against the virus. Additionally, larger classes of BTV-specific small RNAs (26 and 31 nucleotide long) were detected but their role in BTV infection has not been established to date (Schnettler et al., 2013).

1.13 Evolution of BTV

The BTV genome evolves through a combination of genetic drift, reassortment and intragenic recombination (Batten et al., 2008b; Bonneau et al., 2001; He et al., 2010; Nomikou et al., 2015; Shaw et al., 2013).

Analysis of consensus sequence and viral populations of VP2 and NS3 coding segments during alternating cycles of BTV replication in *Culicoides* and a sheep or *Culicoides* and a calf showed that these segments evolve by genetic drift (Bonneau et al., 2001). Although the consensus sequences of both segments remained stable throughout the transmission cycle, heterogeneic populations of virus variants were found in the vector and in the ruminant host. Interestingly, a specific minority variant present in the BTV quasispecies in sheep was selected and fixed in a midge that fed on the animal at 8 d.p.i. Founder effect might therefore play an important role in genetic diversification of BTV strains in nature. In addition, individual genome segments were shown to evolve independently of one another creating a very heterogeneous mutant population both in the ruminant and in *Culicoides* (Bonneau et al., 2001). Furthermore, following the introduction of a new strain into a region, some of the BTV segments (e.g. S3, S10) evolve through strong negative selection. The strains, which emerged and evolved within a

specific episystem, cluster together within one "topotype" (Bonneau et al., 1999; Nomikou et al., 2009). On average BTV segments show between 0.5 and $7x10^{-4}$ nucleotide substitutions per year. S6 (VP5) has been shown to have particularly low substitution rate, likely reflecting the structural constraints imposed by its interaction with the host cell (Carpi et al., 2010). Interestingly, an estimation of the time to the most recent common ancestor (TMRCA) showed that S10 had a mean TMRCA of only 517 years, which was almost ten times less than S2, which has a similar mean nucleotide substitution rate. This evidence of selective sweep that occurred at some point in the last century suggests that BTV underwent a rapid adaptation event that required selection of specific NS3 variants (Carpi et al., 2010). A recent study examining a larger set of BTV sequences showed that multi-dimensional scaling plots of the TMRCA of S1, S3, S4, S5, S8 and S9 clustered closely together while the TMRCA of S2, S6, S7 and S10 did not overlap with the other segments (Nomikou et al., 2015). Based on these data, the authors suggested that S2, S6, S7 and S10 had fewer restrictions imposed by interactions with the remaining segments and could therefore evolve independently of the rest of the genome (Nomikou et al., 2015).

He and colleagues analysed almost 700 BTV sequences in order to determine whether homologous recombination events occurred during virus evolution (He et al., 2010). Signatures of intragenic recombination were found in over 1.6% of the BTV genome. The mosaic genes containing nucleotide fragments of divergent strains were particularly common in segments 1, 7 and 10. The highest number of mosaic elements was found in the last segment, but these never resulted in amino acid changes. Several strains of different spacio-geographical origin seemed to contain genes of the same mosaic ancestor, which might suggest that recombinants might be selected and fixed within populations (He et al., 2010). The exact mechanism or significance of intragenic recombination in BTV is unclear.

BTV reassortment readily occurs in the field and in experimental conditions, and can lead to significant changes in virus pathogenicity and fitness (Batten et al., 2008b; Celma et al., 2014; Coetzee et al., 2014; Maan et al.; Mecham and Johnson, 2005; Shaw et al., 2013). Reassortment events between some serotypes can be flexible and can involve individual or multiple segments at the same time (Shaw et al., 2013). This means that, in theory, if a vector or a ruminant is infected with two strains/serotypes at the same time,

1024 different segment combinations may arise as a result of random reassortment events. However, a large proportion of reassortants do not become established in the population possibly due to functional/structural constrains imposed by specific protein interactions (Nomikou et al., 2015). Several studies attempted to estimate the frequency of reassortment in ruminants or insect vectors (Samal et al., 1987a; Samal et al., 1987b; Stott et al., 1987). Samal *et al.* found that 5% of all viruses recovered from sheep co-infected with BTV-10 and BTV-17 were reassortants (Samal et al., 1987b), while this ratio was almost 90% in infected cattle (Stott et al., 1987). In addition, a high frequency of reassortment was observed in the insect vector and the proportion of reassortants recovered from a single midge ranged from 7% to 78% (Samal et al., 1987a). It is therefore anticipated that multiple reassortant strains may be found in areas where more than one serotype circulate, and it serves as a natural means for rapid genetic shift and generation of novel BTV strains. Reassortment can however be an artificial driver of virus evolution, when field strains recombine with viruses of laboratory origin, and in particular, MLV strains (Chong et al., 2010; Rose et al., 2013). In fact, in 2008, the two South African vaccine strains BTV-6 and BTV-11 were detected in Northern Europe, which were likely introduced through illegal vaccination or transport of immunised animals from outside Europe (De Clercq et al., 2009; Maan et al., 2010). Moreover, reassortants containing segments of MLV BTV-6, BTV-16 and BTV-2 were already reported in the field (Batten et al., 2008b; Maan et al., 2010). Nomikou *et al.* assessed the rates of reassortment of European BTV strains isolated in last 50 years. The authors demonstrated that reassortment was a widespread phenomenon and it was responsible for the high genetic heterogeneity of the European BTV strains. Moreover, at least four MLV strains (South African vaccine strains of BTV-2, -4, -9, -16) were involved in the reassortment events in recent years (Nomikou et al., 2015). These events are of particular concern, as tissue culture attenuation can for instance lead to changes in receptor affinity or tissue tropism (as suggested in the case of transplacental transmission).

1.14 Control of bluetongue

BTV is an economically important disease, due not only to potentially high morbidity and mortality of infected animals, but also because of a decrease in milk and wool production, losses in animal reproduction, and constraints in trade between affected and

unaffected areas. Introduction of a novel strain/serotype into areas with immunologically naïve ruminant populations is of particular concern and warrants imposition of stringent control measures.

Until 1998, the main strategy of bluetongue control was based on "stamping-out", which involved slaughter of all susceptible animals within a 3 km radius of an affected area (Caporale and Giovannini, 2010). Additionally, vaccination provided complementary means to prevent the spread to other areas. In 2000, the World Organisation for Animal Health (OIE) introduced a new directive describing improved bluetongue control procedures based on movement restrictions. Moreover, intensive surveillance, which included clinical and serological assessment of sentinel animals and entomological investigations, were put in place (Caporale and Giovannini, 2010).

In order to facilitate the rapid recognition of BTV-positive animals, diagnostic tests to detect viral antigens or host antibody responses are currently utilised with variable results (Batten et al., 2008a; Eschbaumer et al., 2011; Shaw et al., 2007; Vandenbussche et al., 2008). Serum neutralisation tests are still performed to distinguish between different BTV serotypes, however these are increasingly replaced by nucleic acid amplification based methods or next generation sequencing in the case of newly emerged strains (Maan et al., 2012; Maan et al., 2011). Group reactive qRT-PCR based on genome regions conserved between all BTV serotypes can detect any known viral strain with high sensitivity, while serotype-specific qRT-PCR help with the determination of the causative serotype (Maan et al., 2012; Mayo et al., 2012; van Rijn et al., 2012). ELISA tests have long been a gold standard for the detection of BTV-specific antibodies and currently there are several commercially available kits on the market. Most of these are based on recombinant VP7 proteins and they can efficiently detect humoral responses in animals as early as 7 days after infection (Batten et al., 2008a; Oura et al., 2009). These tests cannot however distinguish between immunised and naturally infected animals, nor between viraemic and non-viraemic ruminants. Similarly, BTV RNA can be detected in blood long after it is possible to isolate the virus, which makes it difficult to distinguish which animals are still infectious (Bonneau et al., 2002; Hoffmann et al., 2009).

Vaccination has long been used as the main means to limit the incidence of bluetongue in endemic areas. Live vaccines hold a particular place in the history of BTV control. The first "attenuated" vaccine was generated by Theiler in 1908 through multiple passage of infectious blood in sheep (Theiler, 1908). The vaccine was routinely used for almost 40 years and was generally considered relatively effective in reducing the severity of disease in sheep. However, increasing numbers of complaints from farmers over the insufficient protection and particularly severe reactions to this vaccine prompted investigations into immunological responses of vaccinated sheep to a variety of circulating BTV isolates (Neitz, 1948). The subsequent discovery of several BTV strains and the lack of adequate cross-protection between heterologous serotypes prompted an investigation into alternative systems for BTV attenuation (Neitz, 1948). Theiler's vaccine was subsequently replaced by strains serially passaged in embryonated chicken eggs in the late 40s (Alexander et al., 1947) followed by tissue-culture attenuated strains in the 60s. MLV generated by passage in eggs and in BHK-21 cells are still produced by Onderstepoort Biological Products in Pretoria (South Africa) and are widely used in Africa (Coetzee et al., 2012a). However, with the increasing number of recognised serotypes, inclusion of multiple serotypes was necessary to achieve adequate protection against the majority of circulating viruses (Howell, 1960; Howell et al., 1970). The current vaccine consists of three vials, each containing five different serotypes, segregated based on their *in vivo* replication efficiency (Coetzee et al., 2012a). Modified live vaccines have also been used in Europe (Bulgaria, France, Italy, Portugal, and Spain) (Roy et al., 2009). However, due to the risks associated with MLV teratogenicity, persistence in the field, under-attenuation or possible reversion to virulence, the use of these vaccines has been quite controversial (Dungu et al., 2004; Monaco et al., 2004; Savini et al., 2010). Indeed, there has been a number of cases where immunisation with MLV strains has led to the development of clinical signs and viraemia in inoculated animals (Savini et al., 2010; Veronesi et al., 2010). One of the South African vaccine serotypes, which was shown to cause disease in European sheep breeds, was BTV-16 (Savini et al., 2010). Indeed, in Italy, the vaccine had to be withdrawn from use and attempts have been made to produce a new attenuated vaccine, innocuous to the native breeds (Franchi et al., 2008).

Due to concerns associated with the use of MLVs, these vaccines were withdrawn from the market in Northern Europe and replaced by inactivated virus formulations. The

inactivated vaccines have been used in Mediterranean countries since 2005 and have proven to be safe, efficacious and to provide good protection against homologous BTV serotypes (Eschbaumer et al., 2009; Hamers et al., 2009; Savini et al., 2008; Savini et al., 2007; Wackerlin et al., 2010). Furthermore, inactivated vaccines were successfully used to control recent outbreaks of BTV in Europe (Mellor and Oura, 2008; Szmaragd et al., 2010).

Recombinant BTV vaccines show promising results in experimental settings, with additional advantages of replicating the virus (the backbone) without the risk of reassortment or reversion to virulence (Boone et al., 2007; Calvo-Pinilla et al., 2009b). Additionally, new-generation products, such as virus-like particles, 'Disabled Infectious Single Cycle strains (with lethal mutation in the VP6 gene) or Disabled Infectious Single Animal (not expressing NS3) were shown to produce good serological responses and serotype specific protection without causing detectable viraemia in the vaccinated animals (Celma et al., 2013; Feenstra et al., 2015; Stewart et al., 2012; Stewart et al., 2013). Moreover, novel platforms allow for the rational incorporation of serotype-specific antigens based on currently circulating strains, and incorporation of diverse antigens into multivalent preparations (Feenstra et al., 2015; Nunes et al., 2014).

Chapter 2
MATERIALS AND METHODS

2.1 Cell lines

All mammalian cell cultures were grown at 37^{o}C in 5% CO_2 humidified atmosphere. BHK-21 and BSR cells (a variant of BHK-21 cells, kindly provided by Karl-Claus Conzelmann) were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (p/s). Transfections in BSR cells were performed in DMEM with reduced FBS and no antibiotics. CPT-Tert cells are sheep choroid plexus cells immortalised with simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (Arnaud et al., 2010). CPT-Tert cells were propagated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 1% p/s. CHO, is a cell line obtained from adult Chinese hamster ovary and pgsA-745 (ATCC® CRL-2242™) is a CHO-derived cells line deficient in xylotransferase that does not produce glycosaminoglycans (GAGs). Both cell lines were propagated in Ham's F-12 medium supplemented with 10% FBS and 1% p/s. A549 cells (human adenocarcinoma epithelial cells) and their variant A549/pr(IFN-β).GFP (expressing GFP under control of IFN promoter; kindly provided by Rick Randall) (Chen et al., 2010) were propagated in DMEM supplemented with 10% FBS. KC cells were derived from *Culicoides sonorensis* larvae and grown at 28°C in Schneider's insect medium supplemented with 10% FBS.

2.2 Primary cell cultures

Primary ovine aortic endothelial (OvEC) cells were isolated from aortas harvested from recently euthanized animals as recently described (Varela et al., 2013). Briefly, the aortas were incubated for 2 h at room temperature in DMEM supplemented with 5% FBS, 25ug/ml p/s and 100U/ml nystatin. The aortas were then rinsed with PBS and excess tissue was trimmed off. The vessels were cut longitudinally and the endothelial cells were stripped off the luminal surface by 1 h treatment with 2 mg/ml collagenase in DMEM at 37^{o}C. After incubation, cells were scraped off the aorta slices, washed once with DMEM and re-suspended in human large vessel endothelial cell growth medium (HuVECM, TCS Cellworks) supplemented with antibiotics, 1% human large vessel endothelial cell growth supplement (TCS Cellworks) and 20% FBS and seeded in 12 well plates. Confluent cultures were trypsinised, pooled and further grown in DMEM. Cells were maintained at 37^{o}C in 5% CO_2 and 3% O_2 for a maximum of 3 passages.

2.3 Virus strains

BTV-$8_{NET2006}$ (Pirbright reference collection number NET2006/04) was originally isolated from a sheep naturally infected during the 2006 outbreak in Northern Europe and has been previously described (Caporale et al., 2014; Ratinier et al., 2011). In this study, we refer to this virus as $BTV8_{L.}$ The subscript "L" is used to denote that this virus has a "low" number of passages in cultures. $BTV8_H$ ("H" – "high passage") was obtained following 65 serial passages of the $BTV8_L$ strain in BSR cells followed by plaque purification. BTV-$8_{NET2007(blood)}$ was derived from the spleen of a sheep infected with blood derived from a naturally infected cow in the Netherlands during the 2007 BTV-8 outbreak as already described (van Gennip et al., 2010). Further viruses were isolated *in vitro* from BTV-$8_{NET2007(blood)}$ after 1 passage in KC cells [BTV-$8_{NET2007(1KC)}$], 1 passage in KC and 1 passage in BHK-21 cells [BTV-$8_{NET2007(1KC-1BHK)}$], 1 passage in KC and 2 passages in BHK-21 cells [BTV-$8_{NET2007(1KC-2BHK)}$] as described in 'Isolation and propagation of BTV-8' section. BTV-8_{IT2008} was isolated in 2008 in naturally occurring outbreak of bluetongue in sheep in Italy.

2.4 Reassortant viruses

$RgBTV8_L$ and $rgBTV8_H$ were derived by reverse genetics (see below). Sequences of the genome of $rgBTV8_L$ were identical to $BTV8_L$ while $rgBTV8_H$ encoded proteins identical to $BTV8_H$ at the amino acid level. The genome of $rgBTV8_H$ did not contain silent mutations that occurred during passage in BSR cells of $BTV8_H$. Each reassortant is described with the name of virus that formed its backbone (either $BTV8_L$ or $BTV8_H$) followed by substituted proteins marked with the "L" or "H" subscript to indicate their origin. E.g., $BTV8_L$+$S2_H$ has the backbone of $BTV8_L$ with VP2 from $BTV8_H$.

2.5 Isolation and propagation of BTV-8

Blood and spleen samples were collected from a single sheep infected with BTV-$8_{NET2007}$-positive infectious blood (Caporale et al., 2014). For isolation of BTV from blood samples, chilled RNAse-free water was added to 500 µl of washed blood, gently mixed and incubated on ice for 10 min. Samples were then centrifuged at 12,000 *g* at 4°C and the supernatant was collected and filtered through a 0.2 µm syringe filter. 250 µl of the filtrate was added to 90% confluent KC cells and incubated for 5-7 days. The medium

was then collected and briefly centrifuged to remove cell debris and used for further passaging in BHK-21 cells. For the isolation of BTV-8 from spleen, samples were homogenised using a sterile quartz and pestle, and diluted in 500 µl of sterile PBS + p/s solution. The homogenate was incubated for 1 h at room temperature and then centrifuged at 10,000 *g* for 10 min. The supernatant was collected, filtered through 0.2 µm syringe filter and 250 µl was used to infect 90% confluent KC monolayers as before. Cells were harvested after passage in KC and BHK-21 cells (after one and two passages) and viral dsRNA was isolated (as described in 'Extraction of dsRNA') for next generation sequencing.

2.6 Extraction of dsRNA

Infected cell monolayers in tissue culture medium were collected (when 80% CPE was formed, or after 5-7 d.p.i. for KC cells) and centrifuged for 10 min at 4000 *g.* Tissue culture fluids were stored at 4^{o}C and cells were used for total RNA extraction using TRIzol (Invitrogen) following the manufacturer's instructions. For the isolation of RNA from spleen, the organ sample was homogenised using a sterile quartz and pestle directly in TRIzol and continued using a standard protocol as recommended by the manufacturer. Next, single-stranded RNA was removed from the sample by precipitation with lithium chloride at 4^{o}C overnight. The precipitated ssRNA was discarded and the dsRNA was precipitated with isopropanol in presence of ammonium acetate for 3 h -20^{o}C. The samples were centrifuged for 15 min at 12,000 *g* and the dsRNA pellet was washed twice with 75% (v/v) ethanol and re-suspended in RNAse-free water. DsRNA was used to amplify each of the 10 genomic BTV segments using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) according to the manufacturer's instructions. The resulting PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and stored at -20^{o}C until used.

2.7 Plasmids

Vectors used for the rescue of BTV8$_{L}$ by reverse genetics were obtained following established procedures (Boyce et al., 2008). The RG constructs for BTV8$_{L}$ were generated by Maxime Ratinier as described before (Ratinier et al., 2011). Briefly, BSR cells cultured to 90% confluency in T75 flasks were infected with specified viruses and incubated at

37^{o}C until 80% CPE was formed. Tissue culture fluids were discarded and cells were used for total RNA extraction using TRIzol (Invitrogen) following the manufacturer's instructions. Single stranded RNA was depleted from the sample by precipitation with lithium chloride at 4^{o}C overnight. The precipitated ssRNA was discarded and the dsRNA was precipitated with isopropanol in the presence of ammonium acetate for 3 h at -20^{o}C. Precipitated dsRNA was washed twice with 75% (v/v) ethanol and re-suspended in RNA free water. DsRNA was used to amplify each of the 10 genomic BTV segments using AccuScript PfuUltra II RT-PCR Kit (Agilent) according to the manufacturer's instructions. The resulting PCR products were gel-purified and cloned into pUC57 vector (Fermentas). Each construct contained the T7 promoter located immediately upstream of the viral sequence and a BsaI or SapI restriction site located downstream of the segment.

The full set of plasmids containing nucleotide sequences of the 10 segments of BTV8$_{H}$ were obtained by mutagenesis of BTV8$_{L}$ vectors using QuikChange II Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. Briefly, the primers for mutagenesis were designed using PrimerX (Table 3). Fifty nanograms of plasmid DNA were used as a template for PCR using PfuUltra II Polymerase (Agilent). Following amplification, the PCR reaction was digested with DpnI for 1 h at 37^{o}C to remove template DNA. The PCR product was then purified using QIAquick PCR Purification Kit (Qiagen) and used to transform XL-10 Gold cells (Agilent) according to the manufacturer's instructions. Resulting colonies were screened for the presence of correct inserts. The correct constructs were transformed into Subcloning Efficiency DH5α cells (Life Technologies) and the cells were used for the amplification of the plasmids. Plasmid preparations were purified with HiSpeed Plasmid Maxiprep Purification (Qiagen) kits and fully sequenced before use.

2.8 Reverse genetics

Twenty micrograms of plasmid constructs containing genomic segments of BTV8$_{L}$ and BTV8$_{H}$ were fully linearised using a specific restriction enzyme (SapI or BsaI). The DNA was purified using phenol-chloroform/isoamyl alcohol twice and followed by chlorophorm/isoamyl acohol extraction. Purified plasmid DNA was then precipitated with isopropanol in presence of sodium acetate overnight at -20^{o}C. Precipitated DNA was washed twice with 70% (v/v) ethanol and resuspended in RNAse free water.

Single stranded RNA was produced using *in vitro* transcription mMESSAGE mMACHINE T7 Ultra Kit (Ambion) according to the manufacturer's instructions with minor changes. Two micrograms of linearised plasmid DNA were used as a template for *in vitro* transcription and additional 1.5 µl of T7 RNA polymerase (20U/µl; Ambion) were added to the reaction mix. After 2 h incubation at 37°C, the template DNA was digested with TURBO DNAse 1 (2U/ul) for 20 min at 37°C. The newly transcribed RNA was extracted using acidic phenol-chlorophorm/isoamyl alcohol followed by chlorophorm/isoamyl alcohol. RNA was further purified by gel filtration using Illustra Microspin G25 columns (GE Healthcare) and then precipitated with isopropanol in presence of sodium acetate for 2 h -20°C. After two washes with 70% (v/v) ethanol, RNA was resuspended in RNAse free water.

Monolayers of BSR cells were grown in 12-well plates to reach ~90% confluency. Cells were then transfected twice with sets of purified single stranded RNA using Lipofectamine 2000 (Invitrogen). Briefly, equimolar amounts of segments 1, 3, 4, 5, 8 and 9 were diluted in Opti-MEM I Reduced Serum Medium containing 0.5 U/mL of RNAsin Plus (Promega) and incubated for 25 min with Lipofectamine 2000 diluted in Opti-MEM I Reduced Serum Medium containing 0.5 U/mL of RNAsin Plus (Promega). The RNA–Lipofectamine complexes were then added to the BSR cells and cells were incubated overnight at 35°C. Second transfection was performed using all 10 single stranded RNA segments in equimolar amounts 18 hours later following the same procedure. Four hours after the second transfection the medium was removed and cells were overlaid with 2 ml of Minimal Essential Medium containing 1.5% agarose type VII and 2% FBS or with fresh DMEM and monitored for CPE development. 48-72 h later transfected cells containing reassortant viruses were collected and used to infect BSR cells. After 80% of CPE developed, the media were collected and centrifuged briefly. Supernatants were stored and used as virus stocks for further experiments. Titres of the viral stocks were determined by standard plaque assays in CPT-Tert cells. All reassortants were screened for correct segment combination by Sanger sequencing of short nucleotide fragments spanning the nucleotides mismatched in $BTV8_H$ compared with $BTV8_L$, as described in 'Sequencing of rescued viruses' section.

Table 3. List of primers used in site directed mutagenesis.

Segment /protein	Amino acid mutation	Primer sequence (5'-3')
S1/VP1	D1231N	GTTGACAAAAACGGAAGTCAATGCCATATCGTTGTATTGC GCAATACAACGATATGGCATTGACTTCCGTTTTTGTCAAC
S2/VP2	E16K	CGAATGTATTCCCAGCGAAGCTTTTAGATGGATATG CATATCCATCTAAAAGCTTCGCTGGGAATACATTCG
	N322D	GAAATATTTCGACCGGGAAATGACGAGCGTACCAACATCATGG CCATGATGTTGGTACGCTCGTCATTTCCCGGTCGAAATATTTC
	I327V	GAAATGACGAGCGTACCAACGTCATGGGGGGCGGAGTACATC GATGTACTCCGCCCCCCATGACGTTGGTACGCTCGTCATTTC
	R400V	CCTACCTATGTACCTTACGATTGGAATAGAGAAAGTGATAAG CTTATCACTTTCTCTATTCCAATCGTAAGGTACATAGGTAGG
S3/VP3	T804M	GCTTGCCTTTTAGTTATGATATGAATGAAAAAGGTGGACTATC GATAGTCCACCTTTTTCATTCATATCATAACTAAAAGGCAAGC
S4/VP4	D322N	GCCGGGGGCTGATGCGAATATGTACGAATTAAG CTTAATTCGTACATATTCGCATCAGCCCCCGGC
	T566I	GTCTGGCGCTTATGTCATAGATTTGTTCTGGTGG CCACCAGAACAAATCTATGACATAAGCGCCAGAC
S5/NS1	N226K	CGAAGGCACAACGCAGTGTACTGCTCAGGTTGTTCTGC GCAGAACAACCTGAGCAGTACACTGCGTTGTGCCTTCG
	A456V	GAACGAGATATTGCCTAGAATTAAGAAGGCGATGGACGAG CTCGTCCATCGCCTTCTTAATTCTAGGCAATATCTCGTTC
S6/VP5	F328I	GAACGAGATATTGCCTAGAATTAAGAAGGCGATGGACGAG CTCGTCCATCGCCTTCTTAATTCTAGGCAATATCTCGTTC
S7/VP7	H276Y	GTTTATAGCTTCAGAGATTACACATGGCACGGGTTGAG CTCAACCCGTGCCATGTGTAATCTCTGAAGCTATAAAC
	A328T	CTGTTTTGAGACCTGAGTTTACGATTCATGGCGTAAACCC GGGTTTACGCCATGAATCGTAAACTCAGGTCTCAAAACAG
S8/NS2	A25T	GCTAAAACATTATGCGGAACGATCGCAAAGTTGAGTTC GAACTCAACTTTGCGATCGTTCCGCATAATGTTTTAGC
S9/VP6	T314A	CATATTAGCTTACACGAGCGCGGGAGGGGATGTAAAAACG CGTTTTTACATCCCCTCCCGCGCTCGTGTAAGCTAATATG
S10/NS3	H97Y	GACTGAGACAGATCAAGCGCTATGTGAACGAGCAGATTTTAC GTAAAATCTGCTCGTTCACATAGCGCTTGATCTGTCTCAGTC

2.9 Plaque assays

Virus titrations were performed in CPT-Tert cells by plaque assays. Briefly, cells were seeded in 12-well plates and incubated until 90% confluent. Serial 10-fold dilutions of stock viruses were prepared in DMEM in triplicate. Dilutions were added to the cells and incubated for 1.5 h at 37^{o}C. After adsorption, the wells were washed with PBS and the cells were overlaid with 1.5 ml of semi-solid overlay (Avicel, 1.4%). The plates were incubated for 72 h at 37^{o}C. The overlay was then removed and the cells were washed twice with PBS. The monolayers were then stained with a crystal violet solution to visualise plaques. Individual plaques were then counted and the mean number of plaques obtained from three replicates was used to calculate virus titers expressed as PFU/ml.

Additionally, separate plaque assays were performed to depict plaque sizes produced by rescued viruses in CPT-Tert cells. The assays were performed in 6-well plates using specific virus concentrations and infections were performed as described above. Infected monolayers were overlaid with Avicel, incubated for 48 h and then stained with crystal violet solution.

2.10 End-point dilution assay

End-point dilution assays were performed in 96 well plates. 100 µl of DMEM supplemented with 4% FBS and p/s were added to each well and 11 µl of each virus sample were added to the top wells in quadruplicate or sextuplicate. Serial 10-fold dilutions were made for each sample. BSR cells were harvested from fully confluent T75 flasks and diluted to 1x10^{5}cells/ml in DMEM supplemented with 4% FBS and p/s. 100 µl of the cell suspension were then added to each well. After 4 days incubation, the cytopathic effect in all wells was assessed and the 50% endpoint dilution was calculated according to the method developed by Reed and Muench and expressed as $TCID_{50}$/ml.

2.11 Growth curves

Growth curves in CPT-Tert, OvEC and A549 cells were performed in 12 or 24-well plates. Ninety percent confluent monolayers were infected at 0.01 MOI of selected viruses. The inoculum was discarded after 1.5 h of incubation at 37^{o}C and cells were washed once with cell culture medium. The medium was then replaced and the cells were incubated

for 72 h. Supernatants were collected at 2, 24, 48, and 72 h.p.i. and centrifuged for 5 min at 500 *g* to remove cell debris. To compare growth of selected viruses in CHO and CHO-pgsA 745 cells, infections were performed at MOI 0.01 as described above and supernatants were collected 72 h.p.i only. Samples from collected time points were titrated by end-point dilution assay and titres were expressed as $Log_{10}TCID_{50}$/ml. The assays were performed twice or three times and the mean result was used to plot growth curves of individual viruses with error bars indicating standard deviations.

2.12 Sequencing of rescued viruses

To confirm the identity of the rescued viruses, 400-800 bp fragments of specific segments that contained mutated nucleotides were sequenced by traditional sequencing method. Briefly, total RNA was extracted from virus-infected cells using TRIzol (Invitrogen) according to the manufacturer's instructions. PCR products were generated using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) using BTV-8 specific primers (Table 4). The sequences were obtained using Sanger sequencing.

Table 4. List of primers used for amplification of RNA fragments spanning regions containing $BTV8_L$/$BTV8_H$ mismatches.

Segment/ protein	Primer sequence (5'-3')	Genome position ($BTV8_L$)
S1/VP1	TGATGATTGGCCCACGGGTTAG GTAAGTGTAATGCGGCGCGTG	3331-3944
S2/VP2	TTCACAAGAGGCGGCGTATAC CACTTTCTCTATTCCTATCGTAAGG	572-1230
S3/VP3	GGATGATATTGAGAGGTTTAGGCAG GTAAGTGTGTTCCCGCTGCC	2162-2772
S4/VP4	ATGGCAATGGCGCAATCCTATTTC GTAAGTTGTACATGCCCCCCTC	1414-1981
S5/NS1	GCTGATGATTGGATCGATCCAAATC AATTTGTTCATCGCGAACCAATTTCG	560-1210
S6/VP5	CTTAACCGAGGAGGAAAAACAAATGAG GGATTTGGGGCTGTTGAGTTCG	510-1120
S7/VP7	ACAGCAGATATTTCAGGGTCGTAAC GTAAGTGTAATCTAAGAGACGTTTGAATG	530-1156
S8/NS2	GTTAAAAAATCCTTGAGTCATGGAGCAAAAG TTTAGCCTCTTCATCCACTTTTGCTTC	1-610
S9/VP6	AGATGAGGTCCCAGTACAGATC GTAAGTGTAAAATCGCCCTACGTC	480-1049
S10/NS3	GTTAAAAAGTGTCGCTGCCATGC CACTCATATCGCTTGAAAGGGTAC	1-452

2.13 Deep sequencing of $BTV8_H$

Full genome sequence of $BTV8_H$ was obtained by Illumina sequencing. Sample preparation and genome amplification were performed by Salvatore Gulletta. Briefly, BSR cells were infected with viruses and total RNA was extracted from the cells using TRIzol Reagent (Invitrogen). Single stranded RNA was precipitated using lithium chloride and double stranded RNA was harvested from the supernatant by precipitation with isopropanol in the presence of sodium acetate. Double stranded RNA was used as a template for full-length amplification of cDNA (FLAC) by reverse transcription PCR (RT-PCR) using the method developed by Maan *et al*. (Maan et al., 2007b). Samples were analysed using the Illumina Genome Analyser (Sir Henry Wellcome Functional Genomics Facility, University of Glasgow). The libraries were constructed from the PCR samples using TruSeq DNA sample preparation kit (Illumina) according to the the manufacturer's instructions. Briefly, DNA samples were fragmented, fragment-end repaired and 3' ends were adenylated. After adaptor ligation steps, the fragments were purified by size selection on agarose gel and the fragments containing adaptors on 3' and 5' end were enriched by PCR. Sequencing was performed on GAIIX sequencer (Illumina) according to manufacturer's protocol. Genomes were assembled using Maq software (Li et al., 2008) with $BTV8_L$ used as a reference sequence. The assemblies were manually curated using Tablet (The James Hutton Institute) for sequence visualisation (Milne et al., 2010) and consensus sequences were generated as fasta files.

2.14 Sequencing of BTV-8_{IT2008}, BTV-$8_{NET2007(blood)}$, BTV-$8_{NET2007(1KC)}$, BTV-$8_{NET2007(1KC-1BHK)}$, and BTV-$8_{NET2007(1KC-2BHK)}$

DsRNA was extracted from the spleen or infected cells as described in "Extraction of dsRNA" section. Full-length genome segments were amplified from dsRNA using the SuperScript III One-Step reverse transcription (RT)-PCR system with Platinum Taq DNA polymerase (Invitrogen) using primers complementary to the 5′- or 3′-end terminus of the viral genome segments (Table 5). The genome of BTV-8_{IT2008} was sequenced using the Sanger method. For the other viruses, equimolar, purified PCR products of the 10 genomic segments of each virus were pooled and sheared by focused sonication (Covaris), followed by size selection using Ampure XP magnetic beads. Illumina MiSeq libraries were generated using the KAPA real-time library preparation kit (KAPA), further

quantified using quantitative RT-PCR (qRT-PCR; KAPA), and sequenced using an Illumina MiSeq with a 300-cycle cartridge as suggested by the manufacturers. Analysis of genetic diversity was carried out using CLC Genomic Workbench version 6.0.1 (CLC bio). After quality assessment and the removal of sequencing artefacts, reads were mapped using BTV-$8_{NET2006}$ as a reference sequence, and the consensus sequences were extracted. Reads with a similarity fraction below 70% were omitted in the final assembly. Single nucleotide polymorphisms were identified using the quality-based variant detection function within CLC Genomics Workbench version 6.0.1. Total sample reads were mapped to the consensus sequence of each segment, and variants were called using, as parameters, nucleotides with total coverage of over 100 reads and a central quality score of Q20 or higher. Average quality score per nucleotide was above Q35.8 in all samples. The mean depth of coverage per variant in each viral genome was between 8,154 and 12,461 reads. Presence of both forward and reverse reads was required to call a variant, while the frequency threshold was arbitrarily set at 0.1%.

Table 5. Primers used to amplify full-length genomic segments of BTV-8.

Segment	Primer sequence (5'-3')
S1	GTTAAAATGCAATGGTCGCAATCACC GTAAGTGTAATGCGGCGCGTG
S2	GTTAAAATAGCGTCGCGATGGAG GTAAGTTGATAGCGCGCGAGC
S3	GTTAAATTTCCGTAGCCATGGCTG GTAAGTGTGTTCCCGCTGCC
S4	GTTAAAACATGCCTGAGCCACAC GTAAGTTGTACATGCCCCCCTC
S5	GTTAAAAAAGTTCTCTAGTTGGCAACC GTAAGTTGAAAAGTTCTAGTAGAGTGCTA
S6	GTTAAAAAAGCGATCGCTCTCGC GTAAGTGGAAAGCGGTGGCTC
S7	GTTAAAAATCTATAGAGATGGACACTATCG GTAAGTGTAATCTAAGAGACGTTTGAATG
S8	GTTAAAAAATCCTTGAGTCATGGAGCAAAAG GTAAGTGTAAAATCCCCCCCC
S9	GTTAAAAAATCGCATATGTCAGCTGC GTAAGTGTAAAATCGCCCTACGTC
S10	GTTAAAAAGTGTCGCTGCCATGC GTAAGTGTGTAGTGTCGCGCAC

2.15 Experimental infection of mice

Transgenic mice deficient in type I interferon (IFN) receptor (129sv IFNAR$^{-/-}$) were maintained at biosafety level 3. For each experiment, groups of adult mice matched for sex and age (n=5 per group) were used.

2.15.1 Survival plots and LD_{50}

Mice were infected intraperitoneally with specified doses of virus or mock-infected with cell culture medium. For *in vivo* pathogenicity studies of parental, reassortant and intermediate passage viruses, doses of 300 PFU and 3000 PFU were used as stated in the Result section. Doses of 5, 10, 30, 100, 300 PFU of rgBTV$_L$ and 300, 1x10^3, 3x10^3, 1x10^4, 3x10^4, 1x10^5 PFU for rgBTV8$_H$ were used in additional experiments. Mice were examined for clinical signs daily until the experiment was concluded 14 days later.

2.15.2 Viraemia, rectal temperature and body weight changes

Groups of IFNAR$^{-/-}$ mice were inoculated intraperitoneally with 300 PFU rgBTV8$_L$, rgBTV8$_H$ or mock infected with tissue culture medium. All mice were micro-chipped and individual mice were monitored for daily changes in body weight and rectal temperature. Blood samples were collected from mice infected intraperitoneally with 300 PFU of rgBTV8$_L$, rgBTV8$_H$ or mock-infected animals at time points indicated in the Result section and blood samples were analysed by qRT-PCR for the presence of viral RNA.

2.15.3 Immunohistochemistry

Spleen, lung and heart samples were collected at various time points p.i. (3, 5 and 9 d.p.i.) after subcutaneous inoculation of IFNAR$^{-/-}$ mice with 300 PFU of rgBTV8$_L$ and rgBTV8$_H$ and from mock-infected animals. Formalin-fixed and paraffin-embedded tissue sections were used in immunohistochemistry. Sections were examined for the presence of BTV using a polyclonal NS2 antiserum and the EnVision (DAKO) detection system according to the manufacturer's instructions.

2.16 Serum neutralisation assay

Sera were collected from mice infected with 300 PFU of rgBTV8$_H$ or mock infected with tissue culture medium at 16 d.p.i. and used in serum neutralisation assays. At 20 d.p.i.,

surviving mice were challenged with 300 PFU of rgBTV8$_L$ and monitored for a further 14 days. Survival plots were constructed for each experimental group.

The presence of neutralising antibodies in infected mice was assessed by neutralisation assays testing serial 2-fold dilutions of sera as previously described (Caporale et al., 2014). Sera were inactivated at 56^{o}C for 30 min prior to testing. Two-fold serial dilutions of each serum sample (total volume 50 µl) and 50 µl of BTV-8 diluted to 100 TCID$_{50}$/ml were added to 96-well plates. After 1 h incubation at 37^{o}C, 100 µl of Vero cells resuspendedmin minimal essential medium, (MEM) were added to each well and plates were incubated for 6-7 days. Following the incubation period, cells in individual wells were scored for CPE. Titres of neutralising antibodies were determined by endpoint dilution assays and reported as log$_{10}$ of the 50% endpoint (proportionate distance [PD]) of each sample tested in quadruplicates.

2.17 qRT-PCR and qPCR

Levels of viraemia in mice were assessed by qRT-PCR as decribed before (Caporale et al., 2014; Caporale et al., 2011). Red blood cells were lysed with ice cold water for 10 min on ice and centrifuged at 4°C for 10 min at 13,000 × g. Armoured West Nile RNA (Asuragen, USA) was spiked into each sample as internal control of extraction efficiency. Total RNA was extracted using High Pure Nucleic Acid Extraction Kit (Roche, Nutley, NJ) according to manufacturer's instructions. For all samples, 250 ng of total RNA were amplified by one-step qRT-PCR using primers and probes for BTV segment 5 (NS1). Armoured RNA and β-actin were amplified as control reactions. Samples were analysed using a 7900HT fast real-time PCR system and the Sequence Detection System Software SDS, version 2.3 (Applied Biosystems). Standard curves were generated by amplifying known concentrations of ***in vitro*** transcribed synthetic BTV segment 5 RNA synthesised using mMESSAGE mMACHINE T7 Ultra kit (Ambion). BTV genome copy numbers were expressed as log$_{10}$/µg of total RNA and threshold cycle (C_T) values ≥40 were considered negative.

Levels of *GAPDH, β-Actin, IFN-β, Mx1, RSAD2* expression in infected OvEC cells were measured by qPCR. Briefly, cells were seeded in 24-well plates and infected 48 h afterwards with high MOI of rgBTV8$_L$, rgBTV8$_H$ and selected reassortants. The medium was replaced after 1 h and the cells were incubated for a further 17 h at 37^{o}C. Next, the

supernatants were collected and monolayers were directly lysed in 0.5 ml of TRIzol (Life Technologies) followed by storage at -80^{o}C until extraction. Phase separation was performed according to the manufacturer's instructions, whereupon the aqueous phase was mixed with ethanol and purified using the RNeasy Mini kit (Qiagen), including RNase Free/DNase set on-column DNase treatment step. Residual contaminating genomic DNA was removed using the TURBO DNA-Free™ kit (Ambion) according to the manufacturer's conditions. Reverse transcription was performed using 100 ng of RNA using random hexamers and SuperScript III (Life Technologies) for 1h at 45°C. qPCR was performed using the Brilliant III Ultra-Fast QPCR mastermix reagents (Agilent) and in-house designed primers/probes (sequences available upon request) targeting ovine *GAPDH, β-ACTIN, IFN-β, Mx1, RSAD2.* Samples were run on an Mx3005P PCR machine with rgBTV8$_{L}$-infected cells set as a calibrator. GAPDH was used as the normalising gene against which fold-induction was determined for *IFN-β, Mx1, RSAD2, β-ACTIN.* qPCR of experiment replicates was kindly performed by Andrew Shaw.

Table 6. Primers and probed used for amplification of ovine genes.

Gene	Primer sequence (5'-3')
IFN-β	CTGAGGAGATGAAGCAAG GGTGAGAATATTGAAGATGTG
Mx1	TAGGACCATAGGTAGAATCTTGAC CTTGACGATCATGTAGCCCT
RSAD2	CAGAAGTACGGTGAATATTTGGAC TGTATTCCTTACACCATGTCCTC
B-Actin	GAAGATCAAGATTATCGCTCCTC GCCAGACTCATCATACTCCT
GAPDH	CCTCTCAAGGGCATTCTAG ATTGTCGTACCAGGAAATGAG
Gene	Probe sequence
IFN-β	5'FAM-TGACCAATACGGCATCTTCCTTCC-3'BHQ1
Mx1	5'FAM-CGTCCACAACCTTGTCTTCCGTGCCT-3'BHQ1
RSAD2	5'FAM-TCCTCGCCATCTCCTGTGACAGCTT-3'BHQ1
B-Actin	5'FAM-AGCCTCCGATCCACACCGAGTAC-3'BHQ1
GAPDH	5'FAM-TGCGACTTCAACAGCGACACTCACTCT-3'BHQ1

2.18 IFN protection assays

Measurement of IFN levels in cell supernatants was based on the methods described previously (Varela et al., 2013). Briefly, OvEC cells were seeded in 24-well plates and after 48-72 h infected with selected viruses at MOI of 1. Culture medium was replaced after 1 h and supernatants collected for analysis 18 h after infection. Supernatants were treated for 20 min with UV light in order to inactivate infectious virus. CPT-tert cells were seeded in 96-well plates and 24 h later serial dilutions of UV-treated supernatants were added to the cells. Serial dilutions of known concentration of universal interferon (UIFN) were used as internal controls. After a 24-hour incubation, the supernatants were removed and cells were infected with encephalomyocarditis virus (EMCV) and incubated for 48 h. Cells were then inspected for EMCV induced cytopathic effect. The levels of IFN in supernatants collected from BTV-infected OvEC cells were calculated based on the number of wells protected from EMCV-induced cell death compared to UIFN control wells.

Cell protection by pre-treatment with universal interferon (UIFN) was performed using CPT-tert cells. Cells were seeded in 24-well plates and 24 h later treated with 1000 units of UIFN. After 18 h incubation, UIFN was removed and cells were infected with selected viruses at MOI 0.01. In parallel, untreated CPT-tert cells were infected with the same set of viruses. The inocula were replaced 1.5 h later with fresh tissue culture medium. At 48 h.p.i, supernatants were collected and virus titrated by end-point dilution assays as described above. At 72 h.p.i., cell monolayers were washed with PBS and stained with crystal violet in a formaldehyde solution to visualise plaques.

2.19 Fluorescence-activated cell sorting (FACS)

A549/pr(IFN-β).GFP cells were seeded in 96 well plates and after 24 h infected with rgBTV8L, rgBTV8H and selected reassortants. For mock-infected samples, tissue culture medium was replaced with fresh DMEM. After 48 h incubation, supernatants were removed and cells were trypsinised and re-suspended to obtain uniform suspensions containing single cells. Samples were then fixed with 4% formaldehyde and FACS was performed to quantify the number of GFP positive cells using the Guava PCA-96 Base System (Merck Millipore) and analysed using InCyte (Merck Millipore). The experiment was performed 4 times in quadruplicate and 4000 events were measured for each

replicate. Images of infected A549/pr(IFN-β).GFP expressing GFP were produced using an EVOS FL Cell Imaging System (AMG, Invitrogen).

2.20 Statistical analysis

All statistical analysis were performed using GraphPad Prism vesion 5.01 (GraphPad Software, Inc., California, USA)

Chapters 3-5
RESULTS

Chapter 3

In vivo and *in vitro* characterisation of BTV-8 extensively passaged in tissue culture

3.1 Introduction

Among the 27 serotypes of BTV found worldwide, at least six serotypes (BTV -1, -2, -4, -8, -9 and -16) have been circulating in different parts of Europe over the last 20 years, each of them showing a marked variation in the degree of pathogenicity (Saegerman et al., 2008). While some serotypes such as the Northern European BTV-8 proved to be extremely virulent, other serotypes, such as BTV-6, -11 and -14, were reported in Europe only on a few occasions and did not cause any clinical signs in the field (Maan et al., 2010; Wilson and Mellor, 2009). Moreover, reassortment between viruses in the field (both wild type and vaccine strains) is extremely common and can lead to the emergence of viruses with a spectrum of different phenotypes (Batten et al., 2008b; Shaw et al., 2013). Additionally, due to other factors such as overwintering and transplacental spread, different serotypes can persist in the environment for many seasons providing an opportunity for genetic drift and the emergence of new strains with altered pathogenicity (Bonneau and MacLachlan, 2004; Bonneau et al., 2001; Gibbs et al., 1979; Maclachlan et al., 2009). Although the concept of BTV serotype/strain related virulence is often quoted in the literature, only a few studies attempted to identify the molecular determinants of BTV pathogenicity are (Caporale et al., 2011; Celma et al., 2014; Waldvogel et al., 1987).

Previously, our group used extensively passaged strains of BTV (including South African life-attenuated vaccines) to identify common genomic segments that are most likely to play a role in BTV virulence (Caporale et al., 2011). With a similar approach, in order to study the molecular determinants of BTV virulence, we have used a BTV-8 strain that was isolated during the bluetongue outbreak in 2006 and had a minimal *in vitro* passage history (BTV-$8_{NET2006}$, referred from now on as $BTV8_L$ in this thesis) and then passaged this virus 65 times in BSR cells and plaque purified ($BTV8_H$). Virulence of both viruses was then assessed *in vivo* in newborn NIH-Swiss mice (intracerebral route) and in sheep. In the mouse model, both viruses caused the same total mortality, however the survival of $BTV8_H$ animals was prolonged by 3 to 5 days (Caporale, unpublished results). In sheep infected with $BTV8_H$ however, we saw no clinical signs of bluetongue while low passage virus was fully pathogenic. Strikingly, the attenuated virus did not induce fever in sheep and we detected no viraemia over the entire course of the experiment (Janowicz et al.,

2015). Furthermore, we detected neutralising antibodies in sheep infected with both viruses albeit the titres were lower in $BTV8_H$ -infected animals (Janowicz et al., 2015).

In this chapter, we characterised genetic differences between $BTV8_L$ and $BTV8_H$ and their phenotypes *in vivo*. $IFNAR^{-/-}$ mouse is a well-established model of bluetongue and the pathological changes observed in these animals infected with wild type BTV viruses resemble those of sheep (Calvo-Pinilla et al., 2010; Caporale et al., 2011; Ortego et al., 2014). Hence, we used this animal model to compare the virulence of $BTV8_L$ and $BTV8_H$ as well as the clinical signs and histopathological presentation induced by both strains.

3.2 Results

3.2.1 $BTV8_H$ shows efficient replication *in vitro* in the absence of a functional IFN system and it is attenuated in $IFNAR^{-/-}$ mice.

First, we assayed *in vitro* replication kinetics of the extensively passaged $BTV8_H$ and the minimally passaged $BTV8_L$ in the ovine cell line CPT-Tert. CPT-Tert cells do not produce IFN and therefore are suitable to assess virus replication kinetics in absence of an IFN-induced antiviral response. Cells were infected with $BTV8_H$ and $BTV8_L$ at MOI 0.01 and virus titres assessed at 2, 24, 48 and 72 h.p.i. Both viruses replicated very efficiently in IFN-deficient CPT-Tert cell lines. However, $BTV8_H$ produced approximately 100-fold higher titres than its minimally passaged equivalent (Figure 6A). The 2-log difference between the two viruses was seen as early as 24 h.p.i. $BTV8_H$ infected monolayers developed full CPE at 48 h.p.i., while it took 72h for $BTV8_L$ to cause total cell death.

Next, we titrated $BTV8_H$ and $BTV8_L$ in IFN-competent primary ovine endothelial cells (OvEC). In these cells, both viruses produced lower yields than in CPT-Tert cells. $BTV8_L$ reached $4.1x10^5$ $TCID_{50}$/ml at 72 h.p.i. (Figure 6B). Strikingly, the titres of $BTV8_H$ peaked at 24 h.p.i. and did not significantly increase over the 72 h period reaching a maximum titre of only $5x10^3$ $TCID_{50}$/ml at 72 h.p.i. These data strongly suggested that through extensive passage in BSR cells, $BTV8_H$ acquired the ability to grow much more efficiently than the original strain in the absence of an IFN response in CPT-Tert cells but was unable to overcome the IFN response in OvEC cells.

In light of these data, we wanted to determine if these diverse phenotypes correlated with differences in virulence in interferon receptor deficient ($IFNAR^{-/-}$) mice. We experimentally infected groups of five $IFNAR^{-/-}$ mice with 300 PFU of each virus intraperitoneally and used tissue culture medium in the mock-infected controls. $BTV8_L$ was highly virulent in this mouse model (100% mortality within 7 days p.i.) while $BTV8_H$ was completely attenuated (Figure 6C). Mice infected with $BTV8_L$ showed signs of BTV infection as early as 2 days p.i. The main symptoms included apathy, ocular discharge and fur ruffling. Additionally, infected mice displayed higher water intake and consequently excessive urination, presumably as a response to increasing body temperature. Mice infected with $BTV8_H$ displayed no visible symptoms of infection throughout the experiment. Complete attenuation of $BTV8_H$ in $IFNAR^{-/-}$ mice clearly

showed that factors other than the IFN response also played a role in the loss of virulence of the tissue-adapted strain.

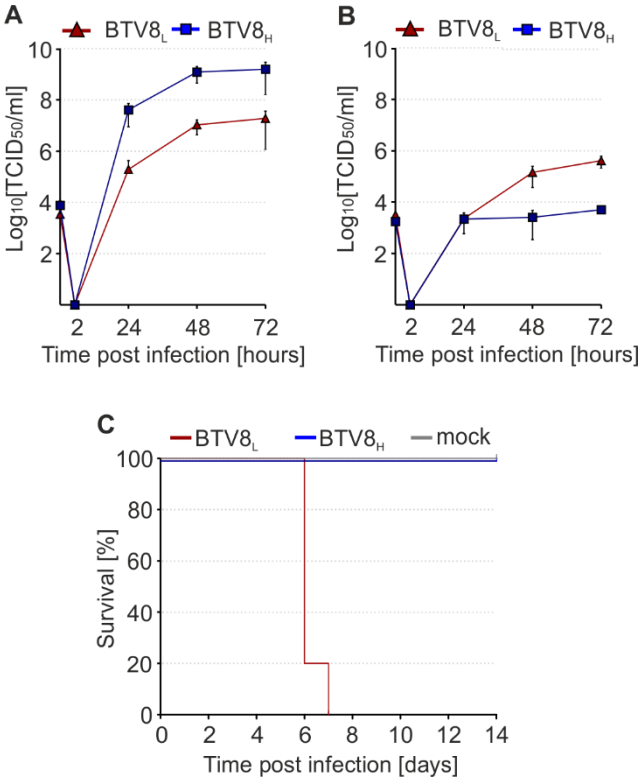

Figure 6. *In vitro* and *in vivo* phenotype of minimally passaged BTV8$_L$ and tissue culture adapted BTV8$_H$. BTV8$_L$ and BTV8$_H$ replication kinetics in ovine CPT-Tert cells (A) and primary ovine endothelial cells (OvEC) (B). Cells were infected with BTV8$_L$ or BTV8$_H$ at MOI 0.01. Supernatants were collected at 2, 24, 48 and 72 h p.i. and then titrated in BSR cells by limiting dilution analysis. Virus titers are expressed as log_{10}(TCID$_{50}$/ml). (C) Survival plot of IFNAR$^{-/-}$ mice (n=5 per group) infected with 300 PFU of BTV8$_L$ and BTV8$_H$ or mock-infected.

3.2.2 Genetic differences between $BTV8_L$ and $BTV8_H$.

Extensive passage of $BTV8_L$ in tissue culture resulted in a virus ($BTV8_H$) possessing a very different phenotype, both *in vitro* and *in vivo*, from the parental strain. To discern which genomic segments played a role in the changed characteristics of $BTV8_H$, we sequenced the full genome of $BTV8_H$ and compared it with the sequence of $BTV8_L$ (Figure 7A). Alignment of both genomes revealed 31 mutations out of which 16 resulted in amino acid substitutions. The non-synonymous mutations were present in each of the 10 genomic segments and affected all proteins except NS4. Several mutations occurred in the non-coding part of the genome and including a GC insertion in segment 6. Interestingly, S2, which is the most variable among the BTV genomic segments in nature (Maan et al., 2004 5075), had the highest number of amino acid mismatches. Two of these, at positions 321 and 327, clustered at the region previously shown to be consistently mutated in tissue culture adapted strains of BTV (Caporale et al., 2011). To study the roles of individual genomic segments in tissue culture adaptation and pathogenicity of BTV8 we first rescued both $BTV8_L$ and $BTV8_H$ by reverse genetics (RG). In order to obtain the plasmids to be used for RG of $BTV8_H$, we used site-directed mutagenesis and introduced each non-synonymous mutation present in $BTV8_H$ into the $BTV8_L$ RG plasmids. Hence, we rescued $rgBTV8_L$ with the same nucleotide sequence as the original virus while $rgBTV8_H$ was identical to the original high passage virus only at the amino acid level.

3.2.3 $RgBTV8_L$ and $rgBTV8_H$ retain phenotypes of the original viruses.

As the genome of the rescued $rgBTV8_H$ only possessed identical viral proteins (but not the nucleotide sequence of genomic segments) of $BTV8_H$, our first goal was to assess whether the amino acid changes introduced were sufficient to confer an attenuated phenotype to the resulting virus. We therefore inoculated groups of five mice with 300 PFU of each rescued virus and monitored the mice over 14 days. Similar to the original $BTV8_L$, $rgBTV8_L$ was highly virulent *in vivo* and caused 100% mortality within the first 4 d.p.i. (Figure 7B). All $rgBTV8_L$-infected mice showed typical signs of infection two d.p.i. with ocular discharge, apathy, ruffled fur and anorexia as the main visible symptoms. As anticipated, $rgBTV8_H$ was fully attenuated and it did not cause any clinical signs of disease. These data showed that both rescued viruses retained the phenotypes of the

original viruses and that amino acid substitutions in $BTV8_H$ were sufficient for the fully attenuated phenotype, at least in $IFNAR^{-/-}$ mice.

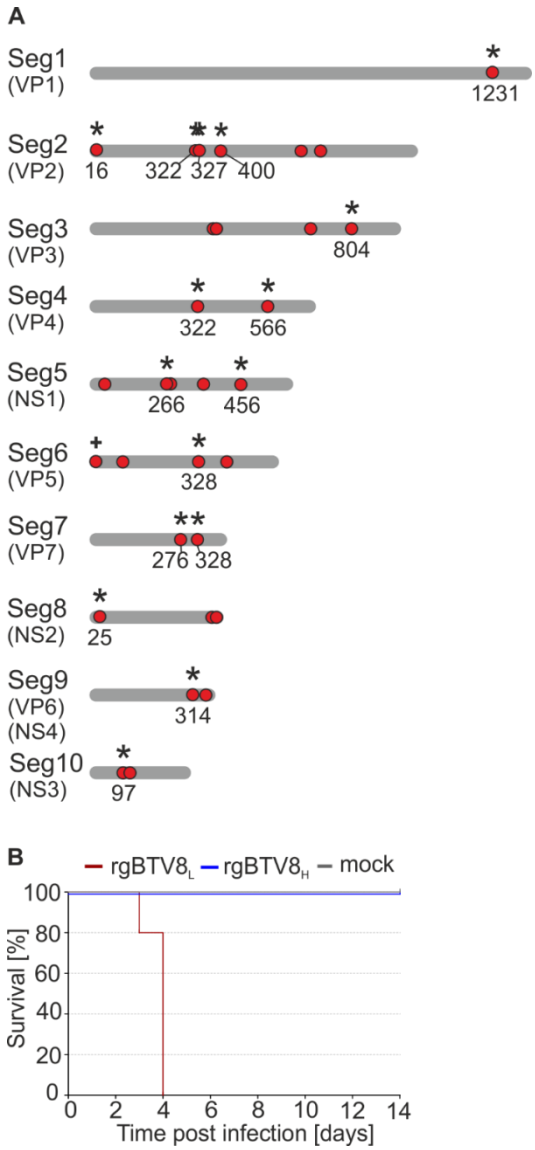

Figure 7. Genetic differences between $BTV8_L$ and $BTV8_H$ and virulence of rescued viruses in $IFNAR^{-/-}$ mice. (A) Schematic representation of the 10 genomic segments of $BTV8_L$ and $BTV8_H$. Mutations in $BTV8_H$ compared to the minimally passaged $BTV8_L$ are indicated with red dots. Non-synonymous mutations are marked with asterisks and the numbers relative to the mutated amino acid residue in the corresponding viral proteins are shown. The plus sign indicates a nucleotide insertion. The length of the schematic genome segments and the relative position of mutations are indicative only. (B) Survival plots of $IFNAR^{-/-}$ mice (n=5 per group) infected intraperitoneally with 300 PFU of $rgBTV8_L$ and $rgBTV8_H$.

3.2.4 Attenuation of rgBTV8$_H$.

A recent study done by Calvo-Pinilla *et al.* showed differences in survival rates of IFNAR$^{-/-}$ mice inoculated with 10 fold dilutions of BTV-4 (Calvo-Pinilla et al., 2009a). At low doses (100 PFU or less) the majority of IFNAR$^{-/-}$ mice infected with BTV-4 survived over 21 days while doses higher than 1x10^3 caused 100% mortality within 7 d.p.i. (Calvo-Pinilla et al., 2009a). Therefore, we wanted to determine if the virulence of rgBTV8$_L$ and rgBTV8$_H$ also correlated to the inoculation dose in IFNAR$^{-/-}$ mice. To this end, we intraperitoneally injected groups of 5 mice with 5, 10, 30, 100 or 300 PFU of rgBTV8$_L$ and 300, 1x10^3, 3x10^3, 1x10^4, 3x10^4 or 1x10^5 PFU of rgBTV8$_H$. RgBTV8$_L$ proved to be highly virulent at all infection doses and the majority of infected mice died within 6 days of inoculation (Figure 8). Only two mice, injected with doses of 5 and 10 PFU, survived infection with rgBTV8$_L$. We did not note any significant differences between the mortality induced by rgBTV8$_L$ at doses of 300, 100 or 30 PFU. Although, two mice inoculated with 300 PFU of rgBTV8$_L$ died at 3 and 4 d.p.i., the majority of animals were died at day 6, similarly to what we observed with doses of 100 and 30 PFU. All mice inoculated with rgBTV8$_H$ survived and remained healthy over the entire course of the experiment, showing that the high passage virus was fully attenuated even at doses as high as 1x10^5 PFU.

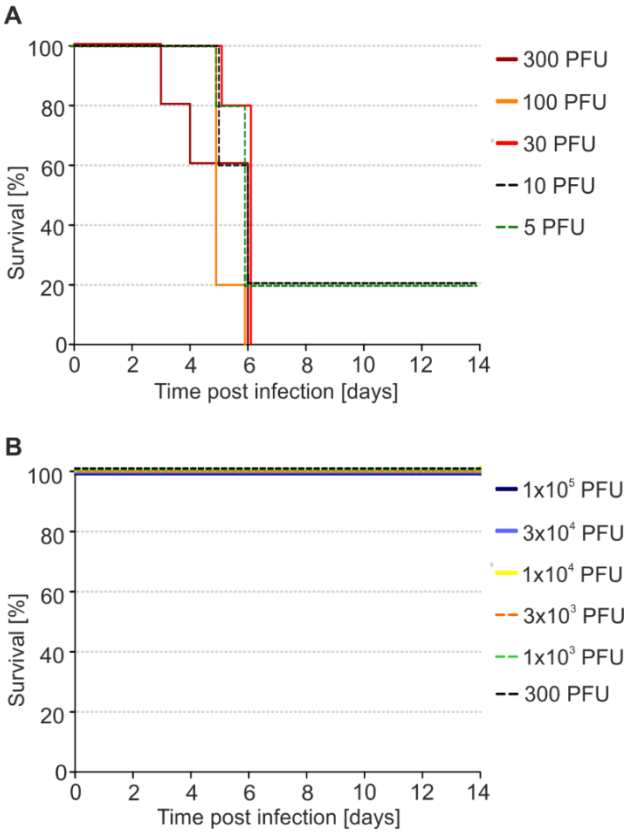

Figure 8. Virulence of rgBTV8$_L$ and rgBTV8$_H$ in IFNAR$^{-/-}$ mice. Groups of IFNAR$^{-/-}$ mice (n=5 per group) were infected intraperitoneally with varying doses of rgBTV8$_L$ (A) or rgBTV8$_H$ (B). Mortality was recorded for 14 d.p.i. Note that rgBTV8$_H$ was attenuated at all inoculation doses.

Although we did not note any characteristic signs of infection, such as ocular discharge or fur ruffling in rgBTV8$_H$-infected mice, we wanted to establish whether the mice displayed subtler signs of infection, such as increase in body temperature, or bodyweight loss. Hence, we micro-chipped groups of five IFNAR$^{-/-}$ mice and inoculated them intraperitonally with 300 PFU of rgBTV8$_L$ or rgBTV8$_H$ or mock infected with tissue culture medium. We measured bodyweight and rectal temperature of individual mice daily over the experimental period of two weeks.

Animals inoculated with rgBTV8$_L$ showed pronounced weight drop starting on the second day after infection (Figure 9B). This correlated with the development of other clinical signs such as apathy and anorexia. Two mice lost over 10% of their body weight 24 h prior to death. In contrast, rgBTV8$_H$ infected mice showed less than 5% average body weight loss over the entire experimental period. The body weight of all mice remained stable for 4 d.p.i. However, a slight decrease was observed (with the exception of one mouse) at 5 d.p.i. Differences were also noted between individual mock-infected mice, where some mice gained and retained more body mass after the inoculation, while one mouse lost weight and did not regained it by the end of the experiment.

The majority of mice infected with low passage virus showed increased rectal temperature as early as 24 h.p.i (Figure 9A). Two of these mice continued to have fever on day 2, which was then followed by hypothermia and death within the next 48 hours. In contrast, the majority of mice inoculated with rgBTV8$_H$ did not develop fever although we detected a slight increase in average rectal temperature at day 6 d.p.i. compared with mock-infected animals. A single mouse in particular showed a rectal temperature approaching 39^{o}C, which was the highest reading we noted in rgBTV8$_H$ infected mice during the course of the experiment. A high degree of variation (up to 1.5^{o}C) was observed in rectal temperatures of mock-infected animals. This suggests that measuring the rectal temperature would not be suitable for detecting subtle rises in body temperature that could be the result of BTV infection.

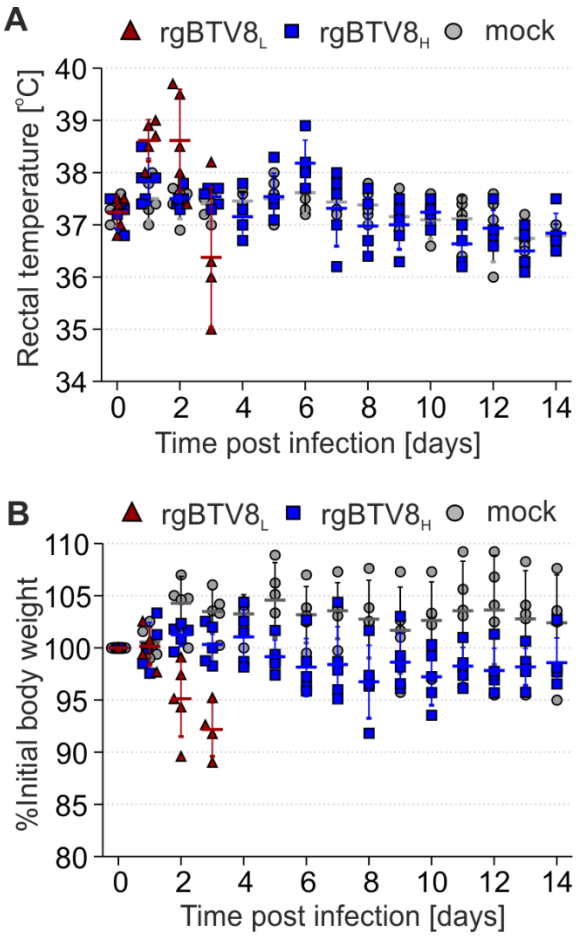

Figure 9. Change in body temperature and body weight following infection with rgBTV8$_L$ or rgBTV8$_H$. Groups of IFNAR$^{-/-}$ mice (n=5 per group) were micro-chipped and infected intraperitoneally with rgBTV8$_L$ and rgBTV8$_H$. Rectal temperature (A) and body weight changes (B) were recorded until 14 d.p.i. Single points represent readings for individual animals. Note that mice inoculated with rgBTV8$_L$ did not survive past 4 d.p.i.

3.2.5 RgBTV8$_H$ induces viraemia and neutralising antibodies in IFNAR$^{-/-}$ mice.

The previous data obtained from sheep experimentally infected with BTV8$_L$ and BTV8$_H$ showed that while BTV8$_L$-infected animals showed high levels of viraemia, BTV8$_H$ RNA was not detected at any time point after experimental infection. Therefore, our next step was to examine whether the same pattern was observed in the mouse model used in this study. We inoculated groups of five micro-chipped IFNAR$^{-/-}$ mice with 300 PFU of rgBTV8$_L$ and rgBTV8$_H$ and collected their blood at the day of inoculation and at 1, 2, 4, 6, 10 and 13 d.p.i. As previously, tissue culture medium inoculated mice were used as controls. Total RNA was extracted from all blood samples and BTV segment 5 RNA was detected by qRT-PCR. BTV RNA was detected in two mice infected with low passage BTV-8 as early as 24 h.p.i (Figure 10). The remaining rgBTV8$_L$-infected mice developed viraemia at 2 d.p.i. The highest BTV RNA load was detected in three mice that survived until day 4. Surprisingly, all mice inoculated with rgBTV8$_H$ also developed viraemia but it was lower (more than 1.5 log lower peak viraemia) and delayed compared with rgBTV8$_L$-infected mice. We did not detect rgBTV8$_H$ RNA until 6 d.p.i., after which time the levels of viraemia started declining. Not surprisingly, the onset of viraemia in rgBTV8$_H$-infected mice coincided with the increased mean body temperature observed in this group of mice. At 13 d.p.i. we were not able to detect any more BTV RNA in three of the five rgBTV8$_H$-infected mice, suggesting that the virus was efficiently cleared from the blood. No BTV RNA was detected in mock-infected control samples.

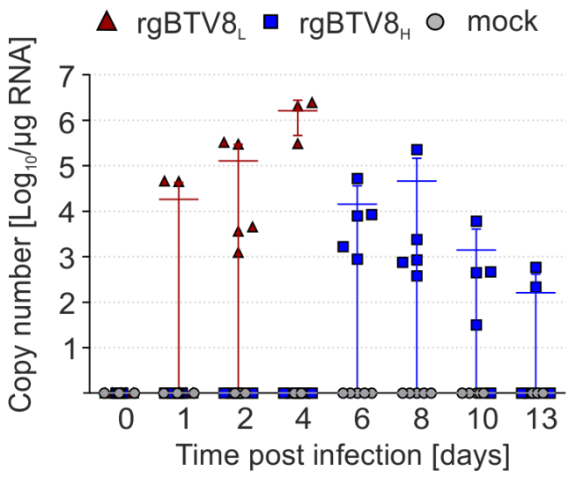

Figure 10. Viraemia in rgBTV8$_L$ and rgBTV8$_H$ infected IFNAR$^{-/-}$ mice Groups of five animals were inoculated intraperitoneally with 300 PFU of rgBTV8$_L$, rgBTV8$_H$ or mock infected with culture media. Blood was collected on the indicated days and viral RNA levels estimated by qRT-PCR as described in Materials and Methods.

Our data suggested that contrary to what we observed in sheep, in IFNAR$^{-/-}$ mice, rgBTV8$_H$ was able to replicate and induce viremia. Our next step was therefore to determine whether mice infected with high passage BTV-8 produced neutralising antibodies and whether infection with the attenuated virus protected animals against challenge with the virulent strain. To this end, we inoculated groups of five IFNAR$^{-/-}$ mice with 300 PFU of rgBTV8$_H$ or tissue culture medium intraperitoneally. We collected sera from all animals at 16 d.p.i. and performed serum neutralisation assays. All rgBTV8$_H$-infected mice developed neutralising antibodies while no antibodies were detected in the sera of mock-infected control animals (Figure 11A). After a 4-day recovery period, mice were challenged with 300 PFU of the virulent rgBTV8$_L$. Mice were monitored for a further 14 days. Control animals challenged with rgBTV8$_L$ developed typical symptoms of bluetongue 2 d.p.i. and 100% mortality was reached 3 days after (Figure 11B). Mice vaccinated with rgBTV8$_H$ survived the challenge and did not develop any clinical symptoms throughout the duration of the experiment. These data confirmed that rgBTV8$_H$ infection led to the development of protective adaptive immune response in IFNAR$^{-/-}$ mice effective against challenge with a virulent strain of BTV-8.

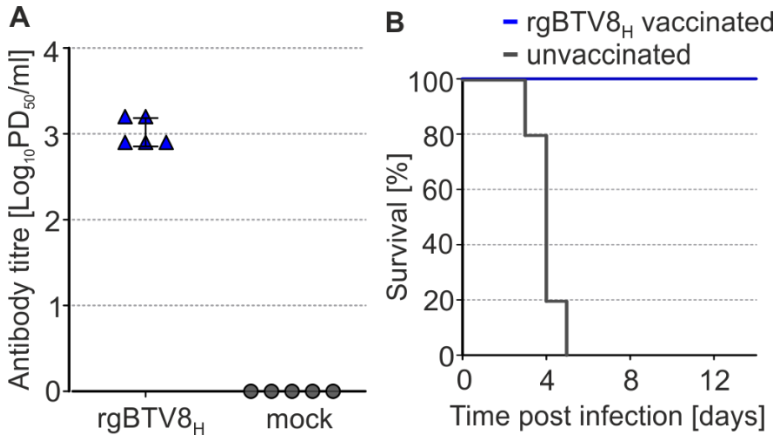

Figure 11. RgBTV8 elicits protective antibody response against challenge with virulent rgBTV8$_L$ in IFNAR$^{-/-}$ mice. Neutralising antibody titres were measured 16 days post infection in sera collected from IFNAR$^{-/-}$ mice (n=5) inoculated with 300 PFU of rgBTV8$_H$ or mock infected with cell culture medium (A). Groups of five IFNAR$^{-/-}$ mice immunised with 300 PFU of rgBTV8$_H$ (rgBTV8$_H$ vaccinated) or inoculated with cell culture medium (unvaccinated) were challenged with 300 PFU of rgBTV8$_L$ and their survival was monitored for 14 days (B).

3.2.6 High levels of rgBTV8$_L$ but not rgBTV8$_H$ are detected in mouse tissues.

We inoculated groups of IFNAR$^{-/-}$ mice subcutaneously with 300 PFU of rgBTV8$_L$, rgBTV8$_H$ or tissue culture medium and collected organs to determine the sites where the virus localised. Previous studies have shown that BTV RNA could be detected in spleen, lung, lymph nodes and thymus of experimentally infected IFNAR$^{-/-}$ mice (Calvo-Pinilla et al., 2010; Calvo-Pinilla et al., 2009a). We therefore collected spleens, lungs and additionally, hearts at 3, 5 and 9 d.p.i. For ethical reasons we used two animals for both infection groups and only one mock infected animal per time point. Gross pathological examination of mice infected with rgBTV8$_L$ and sacrificed 5 d.p.i. revealed widespread oedema, grossly enlarged spleen, distended stomach and slight liver discoloration (Figure 12). No obvious signs of haemorrhage were observed. Pathological changes in animals inoculated with attenuated virus included lung oedema and slight enlargement of the spleen with no discoloration. No changes in stomach size were noted.

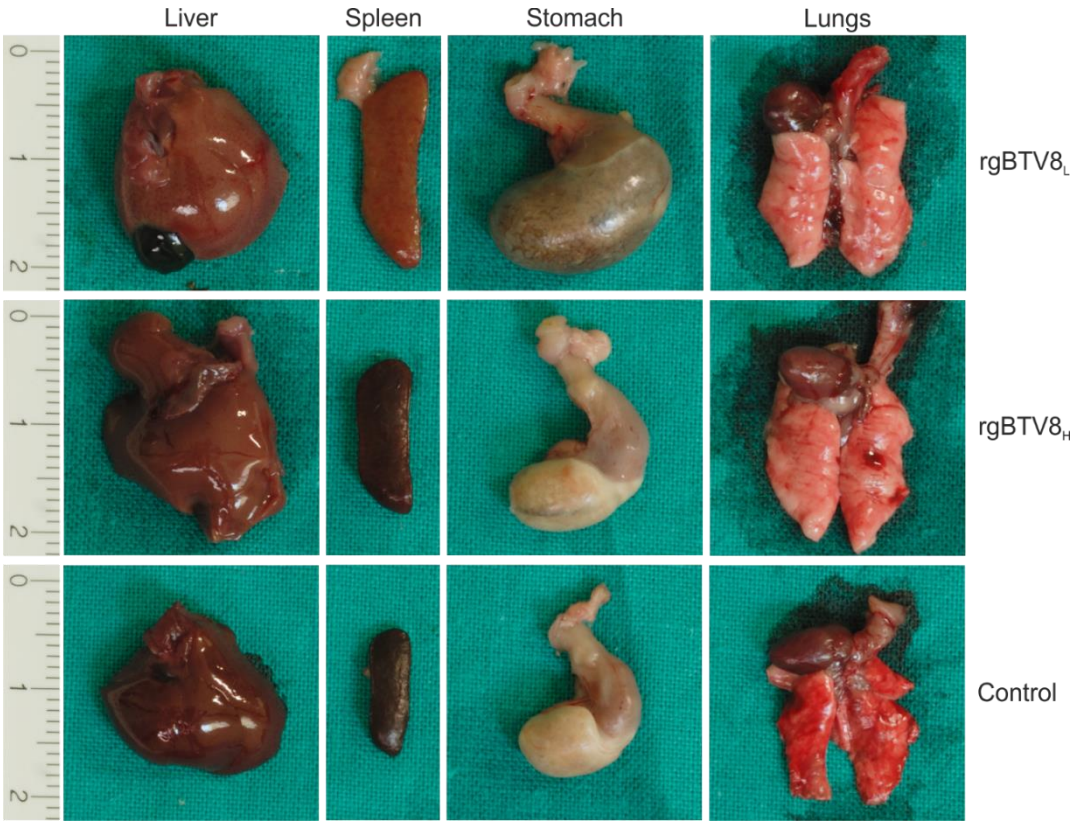

Figure 12. Gross pathological changes in IFNAR$^{-/-}$ mice following infection with rgBTV8$_H$ and rgBTV8$_L$. IFNAR$^{-/-}$ mice (n=5) were inoculated subcutaneously with 300 PFU of rgBTV8$_L$ and rgBTV8$_H$ or mock infected with tissue culture medium (control). Animals were euthanized 5 d.p.i. and their organs (liver, spleen, stomach, lungs) were harvested. Note splenomegaly in rgBTV8$_L$ infected mouse.

Spleens, lungs and hearts harvested from IFNAR$^{-/-}$ mice inoculated with rgBTV8$_L$ and rgBTV8$_H$ at specified time points were fixed in 10% formalin and embedded in paraffin wax. Sections were stained using polyclonal NS2 antiserum and examined for the presence of BTV antigen (Figure 13). The most dramatic effect was observed in the spleen of a mouse infected with the virulent strain (Figure 13 A). At 3 d.p.i. rgBTV8$_L$ antigen was detected across large areas of the spleen, both in white and red pulp. At five days post infection, extensive necrosis was observed and spleen architecture was almost completely erased. The strongest stain was associated with cells surrounding blood vessels. Marked necrosis was also found in the areas where little BTV antigen was found, suggesting that the cell death was related to severe inflammation. In constrast, no lymphoid depletion was observed in spleen samples collected from animals infected with rgBTV8$_H$ at any time point post infection. A few NS2 positive foci were detected 5 d.p.i. and were mainly associated with the white pulp.

Similarly, BTV was only detected in the heart samples of mice infected with BTV8$_L$ (Figure 13B). Only few positive cells were found at 3 d.p.i. but several large areas positive for NS2 were detected at 5 d.p.i. Again, positive cells resembled endothelial cells surrounding blood vessels. BTV was localised in the cardiac muscle. No virus was detected in tissues obtained from mice inoculated with rgBTV8$_H$.

BTV positive cells were only found in lung sections from rgBTV8$_L$-infected mice (Figure 13C). Three days post infection only few cells were detected but the amount increased considerably 5 d.p.i. No virus was detected in lung samples from rgBTV8$_H$-infected mice. Previous experiments showed that viremia was delayed in rgBTV8$_H$-infected IFNAR$^{-/-}$ mice compared with rgBTV8$_L$-infected animals. Therefore, we also collected organ samples from mice inoculated with BTV8$_H$ strains at a later time post infection (9 d.p.i.). No virus was detected by IHC in any of the examined sections at this time point (Figure 14) (as mentioned before mice inoculated with rgBTV8$_L$ die before this time point).

Overall, these results showed that in infected IFNAR$^{-/-}$ mice, rgBTV8$_L$ was able to disseminate efficiently to different organs and replicated mainly in blood vessels associated with specific tissues. In contrast, rgBTV8$_H$ was only transiently found in the follicles of the spleen and it did not seem to cause any marked pathological inflammatory response or necrosis.

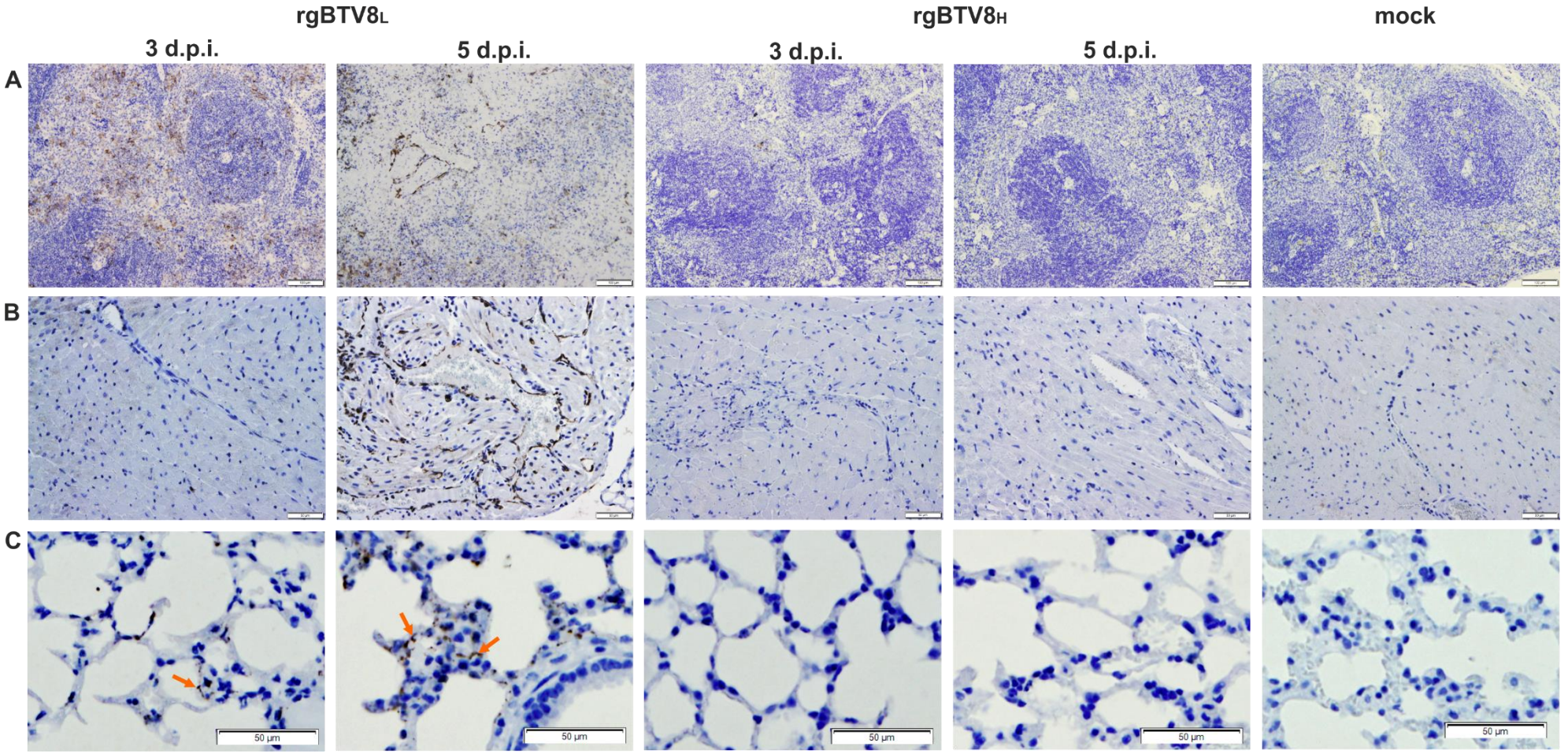

Figure 13. Immunohistochemistry of tissue sections collected from mice infected with rgBTV8$_L$ or rgBTV8$_H$. Mice were inoculated subcutaneously with 300 PFU of the virus or mock infected with tissue culture medium. The mice were sacrificed at 3 or 5 d.p.i. and spleen (A), heart (B) and lungs (C) were collected, formalin fixed and stained to detect BTV NS2. The arrows in (C) indicate BTV positive cells. Scale bars depict 100 µm (A) and 50 µm (B, C).

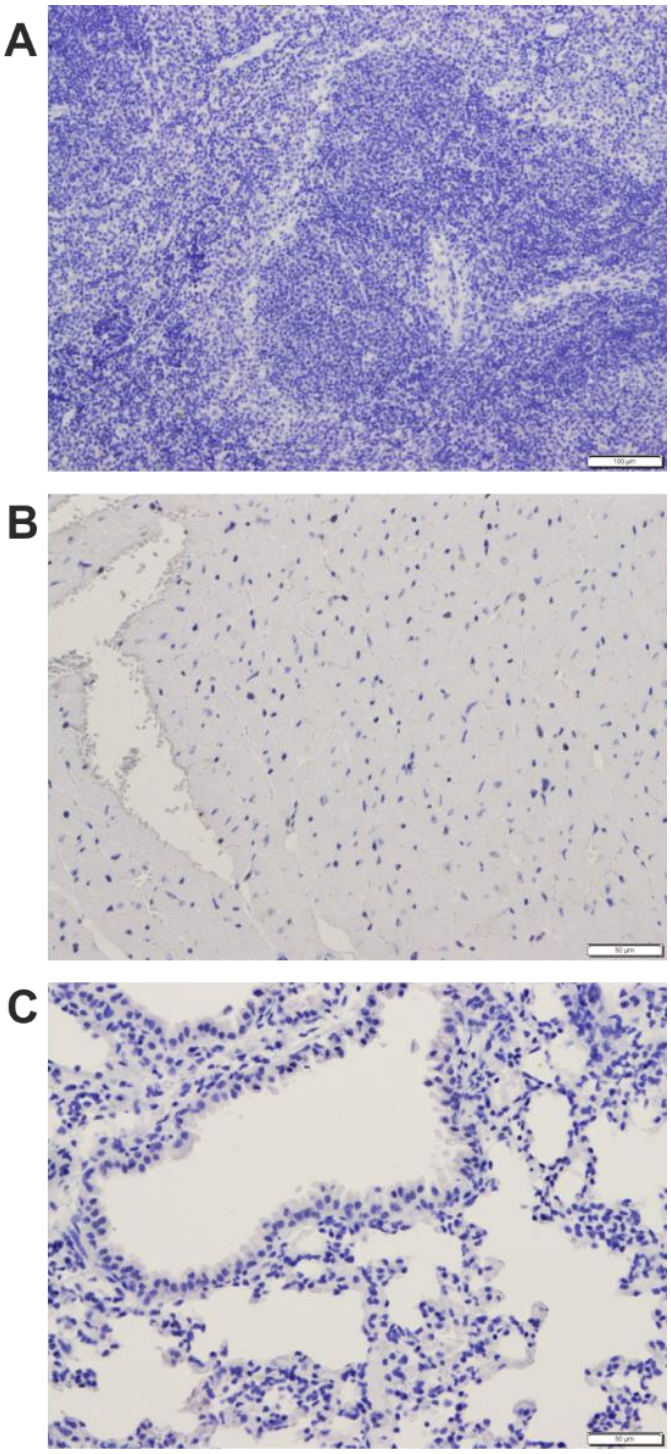

Figure 14. Immunohistochemistry of tissue sections collected from rgBTV8$_H$-infected IFNAR$^{-/-}$ mice. Mice were inoculated subcutaneously with 300 PFU of rgBTV8$_H$ and sacrificed at 9 d.p.i. Spleen (A), heart (B) and lung (C) sections were stained to detect BTV antigen as described in Materials and Methods. Scale bars represent 100 µm (A) and 50 µm (B, C).

3.3 Discussion

In this study, we have developed a strain of BTV-8 that was fully attenuated in sheep. Interestingly, in ovine CPT-Tert cells (an IFN-defective cell line), $BTV8_H$ replicated to titres over 100 times higher than $BTV8_L$. However, replication of $BTV8_H$ was less efficient than $BTV8_L$ in primary ovine endothelial cells. These data showed that the cellular IFN response could play a role in the inhibition of $BTV8_H$ replication. We analysed full genome sequences of $BTV8_L$ and $BTV8_H$ and found 16 non-synonymous nucleotide mismatches between these two viruses, distributed in each viral genomic segment. Rescued $rgBTV8_H$ only contained amino acid changes present in the original virus and the silent mutations were not included in the RG constructs of this virus. Nevertheless, $rgBTV8_H$ retained the phenotype of $BTV8_H$ *in vitro* and *in vivo* in $IFNAR^{-/-}$ mice. We cannot exclude that the silent mutations in the untranslated regions of the genome might have additional effects on the reduced pathogenicity of $BTV8_H$, however, the amino acid mutations were sufficient to fully attenuate the virus at least in $IFNAR^{-/-}$ mice.

Only two small animal models have been used successfully to induce fatal BTV infection (Calvo-Pinilla et al., 2009a; Franchi et al., 2008; Ortego et al., 2014). Intracerebral infection of new-born Swiss-NIH mice has been particularly useful to confirm attenuation of BTV MLV vaccines (Caporale et al., 2011; Franchi et al., 2008). This model is particularly sensitive to BTV. Indeed, our previous work showed that $BTV8_H$ was lethal in newborn NIH-Swiss mice while it is avirulent in sheep and in adult $IFNAR^{-/-}$ mice. Similarly, Caporale *et al.* showed that at least 2 of the South African vaccine strains of BTV that were virulent in 3 year old NIH Swiss mice inoculated intracerebrally, were not pathogenic in $IFNAR^{-/-}$ mice (Caporale et al., 2011). The $IFNAR^{-/-}$ model lacks a functional type I IFN receptor, and therefore animals cannot mount efficient IFN-dependent antiviral responses which renders them highly susceptible to viral infections (Keller et al., 2006; Lorenzo et al., 2010; Ortego et al., 2014). However, the immune system, including the adaptive immune response is otherwise intact and these animals can be used effectively to study various aspects of the pathogenesis of BTV (Caporale et al., 2011; Ortego et al., 2014; Rojas et al., 2011). Here we showed that $rgBTV8_H$ did not cause any apparent signs of disease, although we did detect delayed viraemia in all $rgBTV8_H$-infected animals. These data were in clear contrast with experiments performed in sheep, where no viral RNA was detected in $BTV8_H$-infected animals over the entire course of the experiment. We could

speculate that in the natural host, innate immunity limits the spread and replication of $BTV8_H$, in contrast to $IFNAR^{-/-}$ mice where a functional component of the IFN system is missing, allowing therefore the virus to spread easily to uninfected cells. Moreover, we used the intraperitoneal route of inoculation in mice, while in sheep the intradermal route is used. We therefore could hypothesise that in sheep, $BTV8_H$ is unable to spread beyond the lymph nodes draining the sites of infection, while in IP inoculated mice we would expect to have a wider virus spread. We also observed several day delay in the onset of viraemia in $BTV8_H$-infected mice compared with $BTV8_L$ and viraemia was rapidly cleared during the second week p.i. $IFNAR^{-/-}$ mice infected with virulent strains of BTV did not survive beyond 7 d.p.i. Consequently, a direct comparison of the rate of virus clearance between "wild type" and attenuated strains is not possible in this model. Interestingly, we did not detect $BTV8_H$ in the hearts or lungs of infected mice even at 9 days post infection, and only a few positively stained cells in the spleen at 5 days p.i. This suggests that the attenuated strain was not able to replicate in secondary sites (despite the viraemia) and therefore infection was self-limited, likely cleared by adaptive immune responses. In contrast, high concentrations of $BTV8_L$ antigen were detected in spleen, lungs and hearts of infected mice showing that this strain disseminated to and replicated in these organs rapidly after infection.

Although the $IFNAR^{-/-}$ mouse model does not completely mirror the phenotype of $BTV8_L$ and $BTV8_H$ infection in sheep, it allowed the differenciation between attenuated and virulent BTV strains within a wide range of doses used for experimental infections Hence, this model was suitable to assess the pathogenicity of reassortants between virulent and attenuated strains. Our data showed that mutations acquired through high passage of BTV-8 in an IFN defective cell line led to the loss of pathogenicity of the resulting virus ($BTV8_H$) by several mechanisms. While IFN independent factors were involved in the inability of high passage virus to cause disease in $IFNAR^{-/-}$ mice, other factors, that could have been IFN-related, might have additionally contributed to the observed loss of fitness in OvEC cells and should therefore be assessed further.

Chapter 4

Molecular determinants of BTV-8 virulence

4.1 Introduction

Bluetongue is remarkably variable in its clinical manifestations, which can range from an asymptomatic infection to a lethal haemorrhagic fever (Anderson et al., 1985; Brenner et al., 2011; Maclachlan et al., 2009; Spreull, 1905). This variability is due to a variety of factors related both to the infected host and to the virus (Caporale M., 2014; Maclachlan, 1994; Maclachlan et al., 2009; Oura et al., 2009; Parsonson, 1990; Waldvogel et al., 1987). Over the years, BTV has been used extensively as a prototype virus to study the replication cycle of Orbiviruses, their structural biology and their interaction with the host cell. Although the concept of BTV serotype/strain related virulence is often quoted in the literature, only a few studies addressed the question of what the molecular determinants of BTV pathogenicity are (Caporale et al., 2011; Celma et al., 2014; Waldvogel et al., 1987). Caporale *et al.* showed that MLVs and tissue culture adapted strains of BTV with a history of multiple passages *in vitro* show accumulation of nucleotide substitutions correlating with increasing number of passages in mammalian cells (Caporale et al., 2011). Although specific mutations leading to *in vivo* attenuation of BTV have not been investigated, genomic segments 1, 2 and 8 (encoding VP1, VP2 and NS2) were shown to be consistently mutated in attenuated strains of BTV-2, BTV-4 and BTV-9 passaged extensively in tissue culture (Caporale et al., 2011).

In this chapter, we aimed to determine which genomic segments affect viral fitness *in vitro* and virulence *in vivo.* BTV is a potent inducer of type 1 IFN in sheep and in various cell lines. The ability of the virus to counteract the host IFN response was linked to its pathogenicity (Chauveau et al., 2013; Huismans, 1969; Jameson et al., 1978; Maclachlan and Thompson, 1985; Ratinier et al., 2011; Ruscanu et al., 2012). The data presented in the previous chapter suggested that $BTV8_H$ attenuation might be related to the inability of the virus to counteract the IFN system. Consequently, in our study we investigated genetic factors that contributed to BTV8 virulence either in the presence or absence of a functional IFN system. In $IFNAR^{-/-}$ mice, we assessed the mortality caused by $rgBTV8_L$/$rgBTV8_H$ reassortants and identified segments contributing to attenuation of $BTV8_H$ in absence of IFNAR receptor signalling. Additionally, in IFN competent OvEC we assessed the growth kinetics of selected reassortants, as well as IFN induction and ISG expression in response to infection with these viruses. Our data show that BTV8

adaptation to growth in tissue culture is a complex multifactorial phenomenon related to both IFN-dependent and -independent factors.

4.2 Results

4.2.1 Segment 2 is a major determinant of BTV8 pathogenicity in IFNAR$^{-/-}$ mice.

In the previous chapter, we showed that through extensive passage in tissue culture BTV8$_H$ acquired 16 amino acid changes (compared to BTV8$_L$) and each could be responsible for attenuation of the virus in IFNAR$^{-/-}$ mice. It was however not feasible to create all possible reassortants between BTV8$_L$ and BTV8$_H$. Data published by Caporale *et al.* showed that S1, S2 and S8 are consistently mutated in BTV strains that are attenuated in the mouse model (at least in some strains of BTV-2, -4, and -9). However, their definite role as determinants of BTV pathogenesis has not been established (Caporale et al., 2011). Hence, our first goal was to rescue reassortants with exchanged S1, S2 and S8 (either separately or in combinations), in either the BTV8$_L$ or the BTV8$_H$ backbone, and assess their virulence in IFNAR$^{-/-}$ mice. The set of 14 reassortants was rescued by reverse genetics and titrated in CPT-Tert cells. We then inoculated groups of five mice with either 300 or 3000 PFU of each reassortant and monitored the animals for 14 days. Additionally, we measured weight changes of the mice inoculated with individual reassortants at a dose of 300 PFU.

Mortality plots are shown in Figure 15. We achieved partial attenuation of reassortant viruses by replacing S2$_L$ with S2$_H$. BTV8$_L$+S2$_H$ (i.e. a reassortant containing S2 of BTV8$_H$ within the BTV8$_L$ backbone) caused no mortality at the infection dose of 300 PFU and 40% mortality at 3000 PFU. These data showed that VP2 was one of the main determinants of BTV8 pathogenicity in this mouse model. Only one mouse inoculated with BTV8L+S1$_H$ survived the experiment. However, BTV8$_L$+S1/2$_H$ was completely attenuated at both infection doses. This suggested that the mutation in VP1$_H$ also contributed to BTV8$_H$ attenuation, although its impact was less than that of amino acid changes in VP2$_H$. BTV8$_L$+S8$_H$ and BTV8$_L$+S1/8$_H$ were fully virulent and caused 100% mortality within the first 6 d.p.i. Unexpectedly, we noted 20% mortality of mice inoculated with 300 and 3000 PFU of BTV8$_L$+S1/2/8$_H$. This showed that S8$_H$ did not carry attenuating mutations but, on the contrary, it made the virus more virulent than BTV8$_L$+S1/2$_H$ *in vivo*. None of the reassortants that contained the BTV8$_H$ backbone was virulent in this mouse model, as we noted no mortality or typical symptoms of BTV

infections such as fur ruffling or ocular discharge. These data suggested that attenuation of BTV8 involved mutations in segment(s) other than S1 and S2.

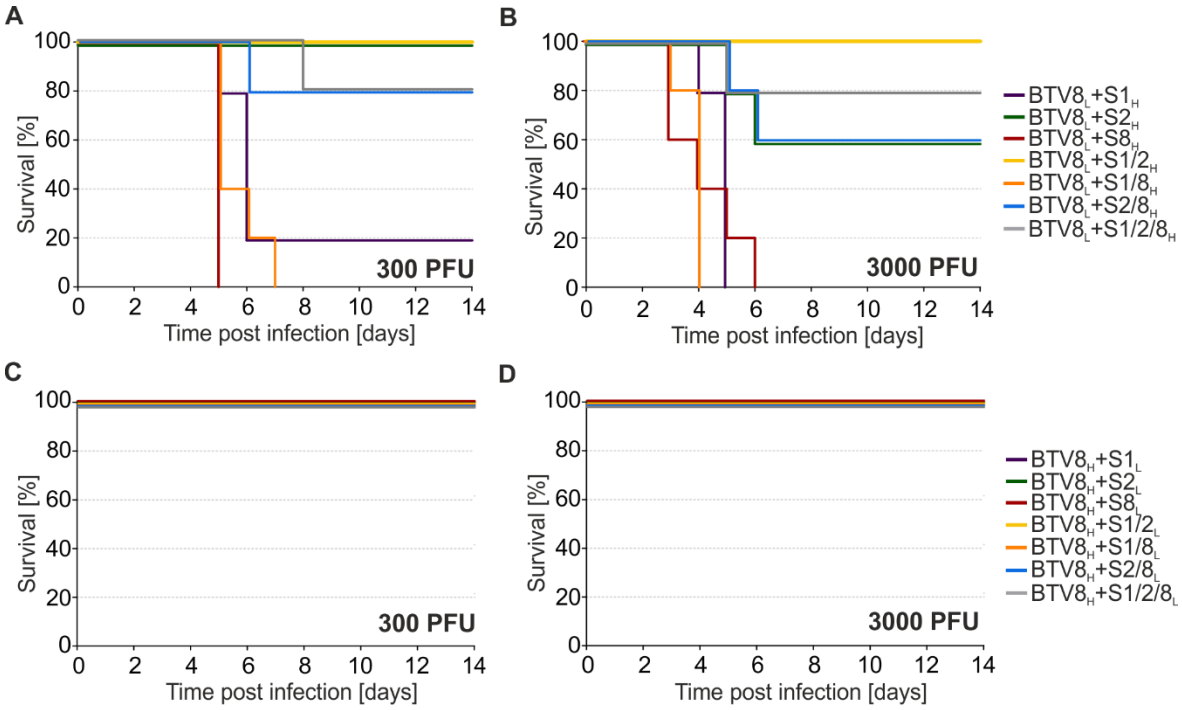

Figure 15. Virulence of S1, S2 and S8 reassortants with $BTV8_L$ or $BTV8_H$ backbone. Groups of 5 $IFNAR^{-/-}$ mice were inoculated intraperitoneally with either 300 or 3000 PFU of $BTV8_L$/$BTV8_H$ reassortants with exchanged segments 1, 2 and 8. Panels show mortality plots of reassortants with the $BTV8_L$ backbone (A, B) or the $BTV8_H$ backbone (C, D). Note that all reassortants in the $BTV8_H$ background were attenuated at both 300 and 3000 PFU inoculation doses.

In addition, we assessed the average weight change in mice inoculated with 300 PFU of each of the 14 reassortants, the parental viruses and in the mock-infected animals. In general, no differences in weight loss were observed in mice infected with reassortant viruses or infected with the parental viruses (Figure 16). An exception included single segment reassortants with exchanged S2. Mice inoculated with $BTV8_H$+$S2_L$ showed more than 5% average weight loss at 6 d.p.i., which was approximately 3% more than we observed in $rgBTV8_H$-infected mice. This difference was however much less pronounced than that observed between animals inoculated with $rgBTV8_L$ (or containing the $BTV8_L$ backbone) and $rgBTV8_H$. Please note that the values indicated are only averages for the distinct groups as mice were not micro-chipped prior to the experiment and therefore we could not discern between weight changes of individual animals nor establish the range of body weight loss at individual time points.

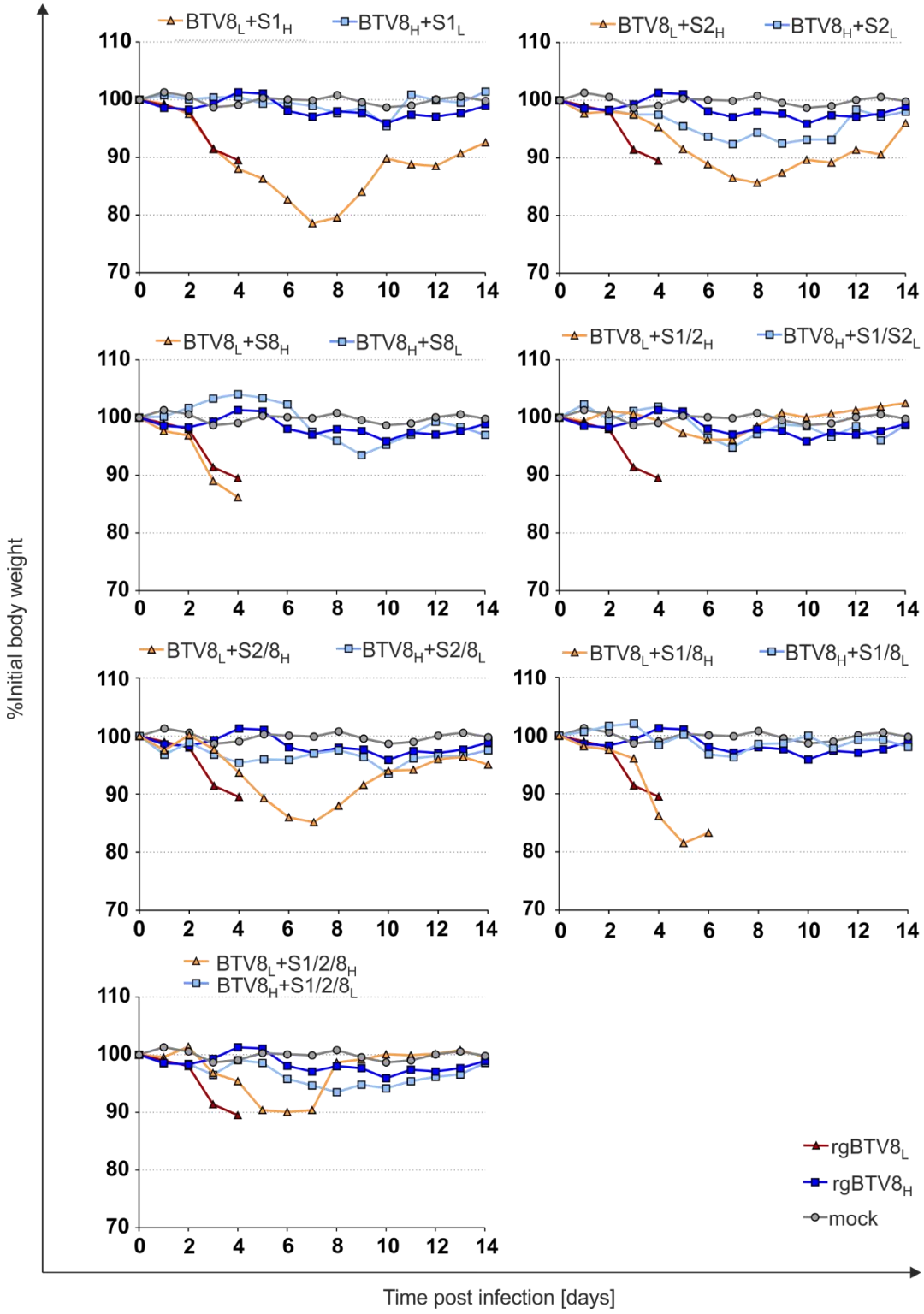

Figure 16. Body weight changes of IFNAR$^{-/-}$ mice following infection with S1, S2 and S8 reassortants with BTV8$_L$ or BTV8$_H$ backbone. Groups of five IFNAR$^{-/-}$ mice were inoculated intraperitoeally with 300 PFU of rgBTV8$_L$, rgBTV8$_H$ or reassortants (as indicated in individual panels). Body weight changes were recorded daily and mean body weight per group (presented as of % of initial body weight) were plotted.

4.2.2 Progressive attenuation of BTV8 during cell culture passages.

The data accumulated so far clearly showed that more than one genomic segment determined the pathogenicity of BTV8 in IFNAR$^{-/-}$ mice. We identified S2 as the main virulence factor and, additionally, S1 as a minor determinant that contributed to BTV8$_H$ attenuation albeit to a lesser extent. Since the mismatches between BTV8$_H$ and BTV8$_L$ occurred in all 10 genomic segments, instead of testing the remaining seven, we attempted to exclude some segments from our analysis. To this end, we analysed genomic sequences and *in vivo* pathogenicity of three viruses collected after 21, 38 and 56 passages of BTV8$_L$ in tissue culture (p21, p38, p56 respectively) and examined how the emergence of amino acid changes in the consensus sequences correlated with decreased mortality in IFNAR$^{-/-}$ mice. Note that BTV8$_H$ had been derived from passaging BTV8$_L$ 65 times in tissue culture. dsRNA was isolated from cells infected with these intermediate passage viruses and used to amplify the ten viral genomic segments. Samples were Sanger-sequenced using fragment-specific primers and sequences were compared to BTV8$_L$. In order to assess virulence in IFNAR$^{-/-}$ mice, groups of five IFNAR $^{-/-}$ mice were inoculated with 300 or 3000 PFU of BTV8 p21, p38 and p55 and their mortality was recorded over a period of 14 days. As expected, virulence of the intermediate passage viruses decreased with the increasing number of passages (Figure 16). Five amino acid mutations present in BTV8$_H$ were found in the consensus sequence of p21 (in S2, S4, S8, S9 and S10). However, we observed double peaks in chromatogram sequences for four of these (S4, S8, S9, S10) suggesting the presence of mixed virus populations that could contain combinations of mutated and original sequences. Moreover, we found two non-synonymous mutations that were not present in BTV8$_H$ (in S2 and S3). *In vivo*, p21 displayed intermediate level of virulence compared to BTV8$_L$ and BTV8$_H$ and caused 60% mouse mortality at 300 and 3000 PFU. Additional amino acid substitutions occurred in the consensus sequence of p38 and these included mutations in all segments except S3. Again, we identified polymorphisms in S1, S5 and S8. Unlike BTVp21 however, BTV8p38 was attenuated *in vivo*. The majority of BTV8p38-infected mice remained healthy throughout the experiment and only one death occurred at 4 d.p.i. Previous experiments showed that BTV8$_L$ is virulent in IFNAR$^{-/-}$ even at very low infection doses. Hence, it is possible that the inoculum used to infect the mouse that did not survive the experiment contained a fraction of viral population that had not acquired attenuating mutations and

as such retained their pathogenic potential. Almost all amino acid substitutions that occurred in $BTV8_H$ were also found in BTV8p56. The only exception was a mismatch in S3 that must have emerged during further passages. As expected BTV8p56 was non-pathogenic in $IFNAR^{-/-}$. It is important to note that while $BTV8_H$ was plaque-purified, BTV8p21, BTV8p38 and BTV8p56 were not, and therefore additional mutations and mixed virus populations were detected in these virus preparations. We cannot exclude that the mismatches and polymorphisms that were not present in either $BTV8_L$ or $BTV8_H$ passage virus also had an effect on virulence of these strains.

To summarise, we observed two levels of reduction of virulence of these intermediate viruses. The first one occurred during the first 21 passages and it was likely attributed to either individual or a combination of mutations in S2, S4, S8, S9, and S10. A further decrease in pathogenicity was observed between p21 and p38 and it could have involved amino acid substitutions in all other segments except S3. However, amino acid substitution in S5 of p38 appeared to be a polymorphism and therefore we concluded that S5 was unlikely to be one of the major factors involved in $BTV8_H$ attenuation. Hence, to refine our approach further, in our next experiment we ruled out S3 and S5, as well as S1 and S8 that had already been tested in the earlier work.

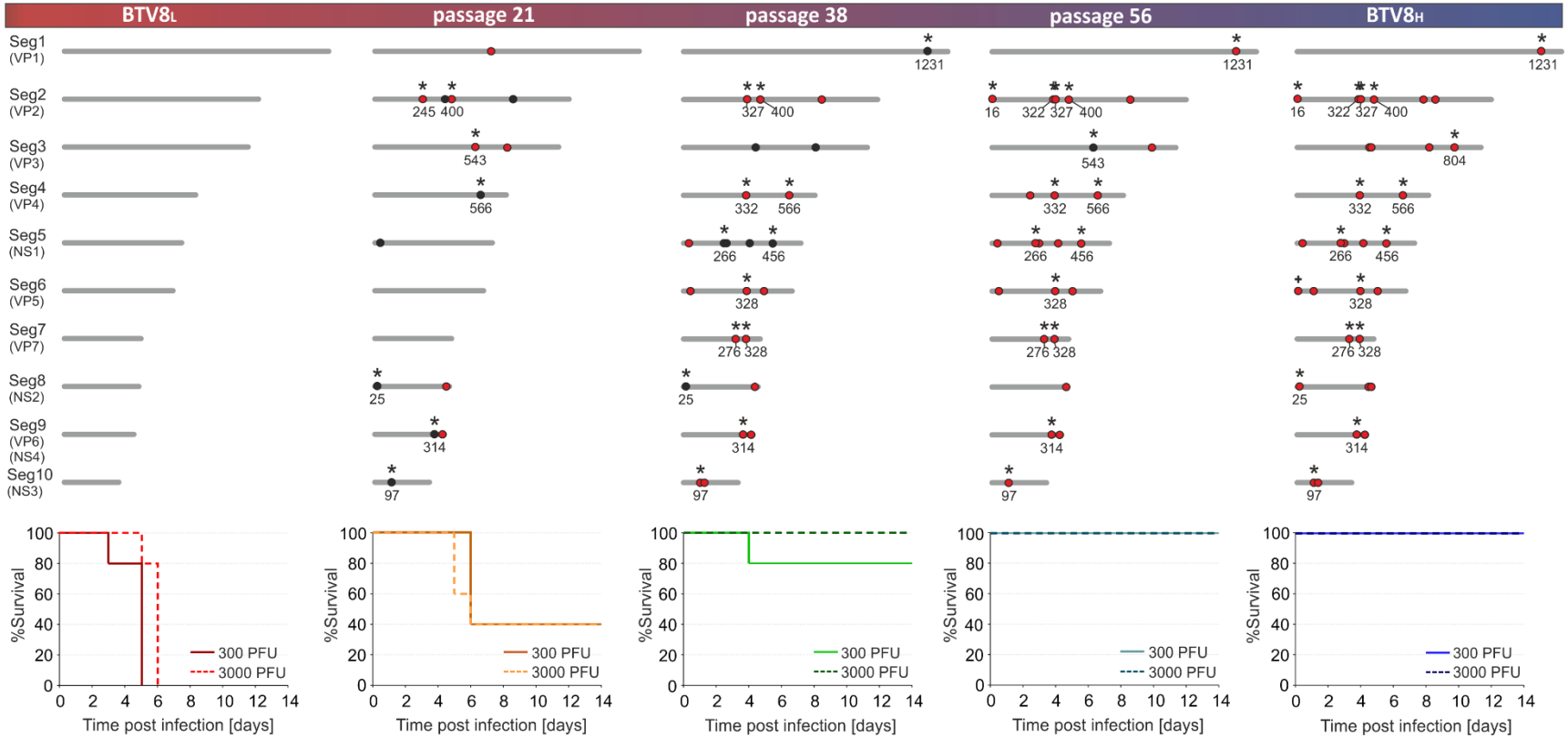

Figure 17. Genetic differences between BTV8$_L$ and BTV8$_H$, and intermediate passage viruses BTV8p21, BTV8p38 and BTV8p65 and their virulence in IFNAR$^{-/-}$ mice. Schematic representation of the 10 genomic segments of BTV8$_L$, BTV8$_H$, BTV8p21, BTV8p38 and BTV8p65. Mutations in the intermediate passage viruses and BTV8$_H$ are compared to the minimally passaged BTV8$_L$ and are indicated with dots. Black dots depict nucleotides where polymorphisms were detected in the sequencing chromatograms and red dots correspond to homogenous bases. Non-synonymous mutations are marked with asterisks and the numbers relative to the mutated amino acid residues in the corresponding viral proteins are shown. The plus sign indicates a nucleotide insertion. The length of the schematic genome segments and the relative position of mutations are indicative only. Survival plots of IFNAR$^{-/-}$ mice (n=5 per group) infected intraperitoneally with 300 PFU of BTV8$_L$ and BTV8$_H$ and intermediate passage viruses.

4.2.3 Complete attenuation in IFNAR$^{-/-}$ mice is achieved by combining S2$_H$ with S6$_H$ or S10$_H$ in BTV8$_L$ backbone.

We previously identified S2 as a major determinant of BTV8 pathogenicity and replacement of S2$_L$ with S2$_H$ lead to marked (although not complete) attenuation in infected IFNAR$^{-/-}$ mice. Our next goal was to find if, in combination with S2$_H$, any other segment would confer full attenuation to the resultant reassortant *in vivo*. Based on analysis of intermediate viruses, we constructed a set of double segment reassortants that contained the BTV8$_L$ backbone and the BTV8$_H$ S2 in conjunction with either S4, S6, S7, S9 or S10 also from BTV8$_H$. Additionally, we rescued the counterpart reassortants containing the BTV8$_H$ backbone in order to find whether exchange of two segments would be sufficient to restore BTV8$_L$ virulence. As previously, groups of 5 IFNAR$^{-/-}$ mice were inoculated intraperitoneally with 300 or 3000 PFU of each of the 10 reassortants and observed for clinical signs over 14 days. Two reassortants with the BTV8$_L$ backbone, BTV8$_L$+S2/6$_H$ and BTV8$_L$+S2/10$_H$ showed complete attenuation at both infection doses, which suggested that S6 and S10 contributed to attenuation of BTV8$_H$ (Figure 18). We observed 0%, 20% and 40% mortality in animals infected with 300 PFU of BTV8$_L$+S2/4$_H$, BTV8$_L$+S2/7$_H$ and BTV8$_L$+S2/9$_H$, respectively. Mice survival decreased with increased infection dose (3000 PFU) to 60%, 40% and 60% for BTV8$_L$+S2/4$_H$, BTV8$_L$+S2/7$_H$ and BTV8$_L$+S2/9$_H$-infected mice, respectively. Surprisingly, none of the viruses with the BTV8$_H$ backbone caused mortality or clinical symptoms in IFNAR$^{-/-}$ mice, which proved that a combination of more than two BTV8$_L$ segments was necessary to confer virulence to a virus containing the BTV8$_H$ backbone.

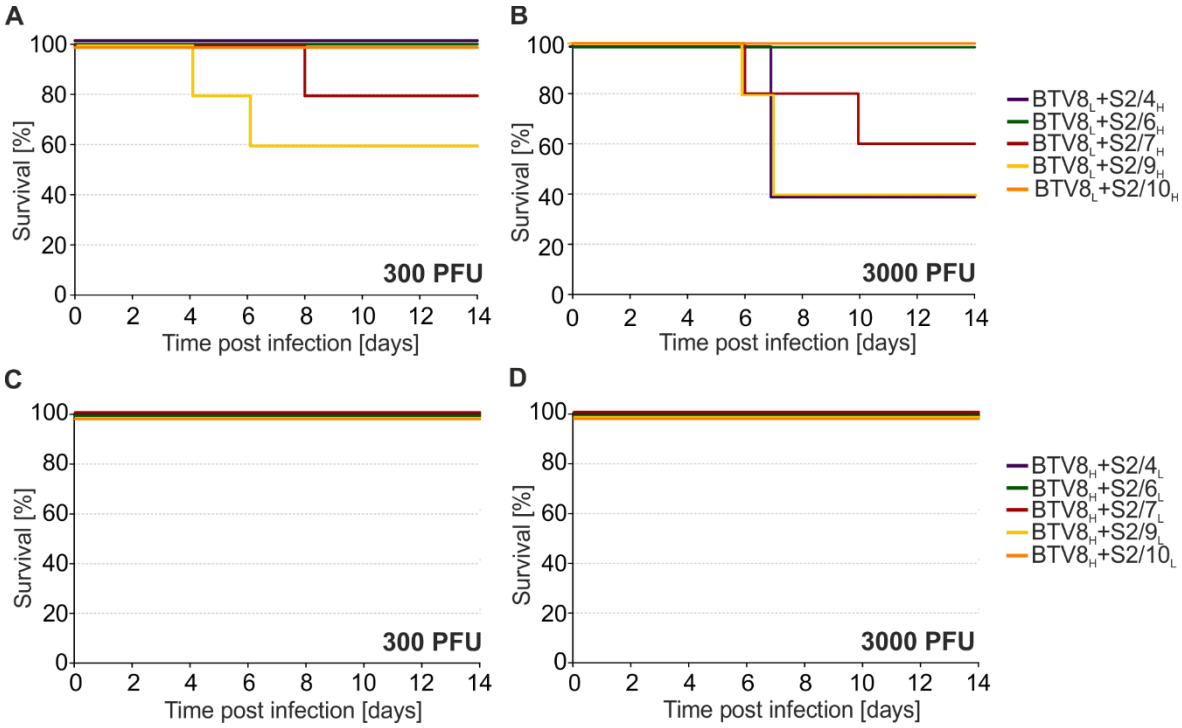

Figure 18. Virulence of double segment reassortants with $BTV8_L$ or $BTV8_H$ backbone. Groups of five $IFNAR^{-/-}$ mice were innoculated intraperitoneally with either 300 or 3000 PFU of $BTV8_L$/$BTV8_H$ reassortants with exchanged segment 2 and either 4, 6, 7, 9 or 10. Separate panels show mortality plots of reassortants with $BTV8_L$ backbone (A, B) or $BTV8_H$ backbone (C, D). Note that all reassortants in $BTV8_H$ background were attenuated at both 300 and 3000 PFU inoculation doses.

4.2.4 A minimum of five BTV8$_L$ segments within BTV8$_H$ backbone are required to restore virulence in IFNAR$^{-/-}$ mice

Although it was possible to attenuate BTV8$_L$ fully by replacing two of its genomic segments by BTV8$_H$ equivalents, we were not able to restore virulence to BTV8$_H$ by double segment replacement. We therefore rescued another set of reassortants containing three or more proteins of low passage virus in the BTV8$_H$ backbone and tested them in the mouse model for increased pathogenicity. Triple segment reassortants were generally non-pathogenic in IFNAR$^{-/-}$ mice (Figure 19). All animals infected with BTV8$_H$+S2/6/7$_L$ and BTV8$_H$+S2/7/10$_L$ survived the experiment and only one mouse inoculated with 3000 PFU of BTV8$_H$+S2/6/10$_L$ died at 7 d.p.i. Surprisingly, BTV8$_H$+S1/2/6/10$_L$ also possessed an attenuated phenotype. These data suggested that other viral proteins besides VP1, VP2, VP5 and NS3 also play a role in the virulence of BTV8$_L$. We found that a tetra-reassortant, BTV8$_H$+S2/6/7/10$_L$ caused clinical signs in mice and 20% and 60% mortality when injected at 300 and 3000 PFU, respectively. To achieve 100% mouse mortality, at either 300 or 3000 PFU inoculation doses, it was required that at least 5 proteins of BTV8$_H$ were replaced by the BTV8$_L$ equivalents, including either VP4 or VP7 in conjunction with VP1, VP2, VP5 and NS3. Hence, mutations in S4$_H$ and S7$_H$ must have also contributed to attenuation of BTV8$_H$, either by having a direct effect on their function *in vivo* or by decreasing structural/functional compatibility between BTV8$_H$ and BTV8$_L$ proteins.

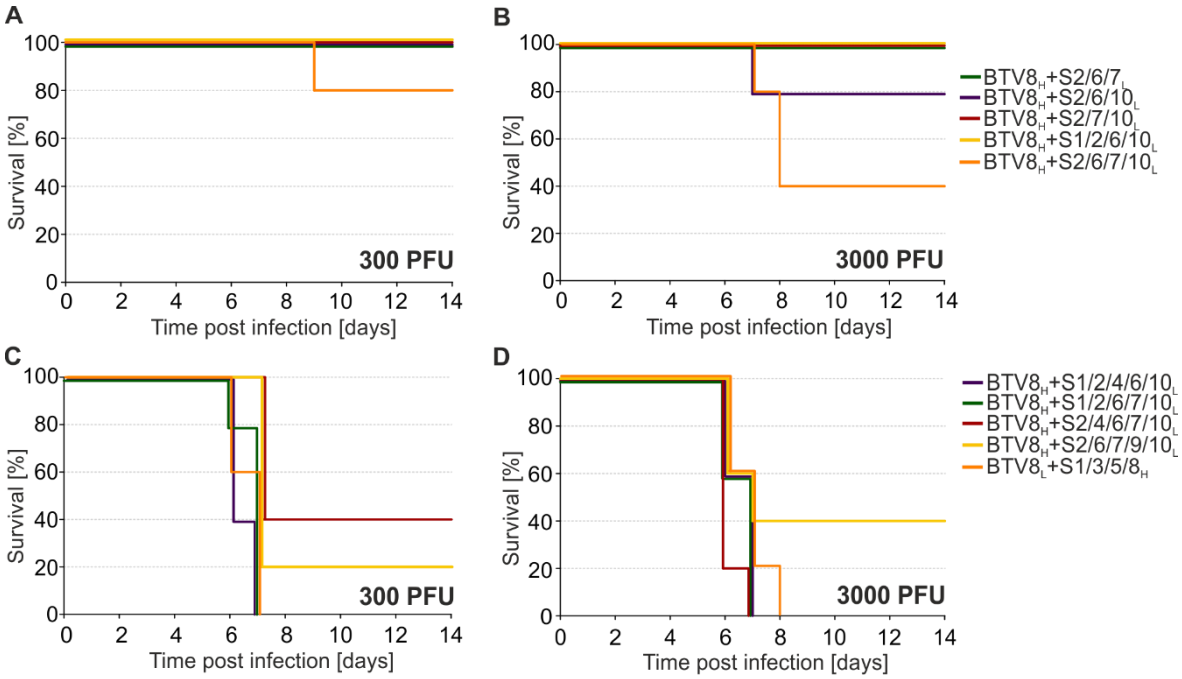

Figure 19. Virulence of BTV8$_L$/BTV8$_H$ reassortants. Groups of 5 IFNAR$^{-/-}$ mice were innoculated intraperitoneally with either 300 or 3000 PFU of BTV8$_L$/BTV8$_H$ reassortants and monitored for 14 d.p.i. Figure was divided into four separate panels that show mortality plots of reassortants with BTV8$_H$ backbone and three or four segments of BTV8$_L$ (A, B) or containing five or six segments of BTV8$_L$ (C, D). Note that full virulence at 300 and 3000 PFU was observed in animals inoculated with BTV8$_H$+S1/2/4/6/10$_L$, BTV8$_H$+S1/2/6/7/10$_L$ and BTV8$_L$+S1/3/5/8$_H$ only.

4.2.5 $BTV8_L+S4_H$ and $BTV8_L+S10_H$, but not $BTV8_L+S6_H$, show partial attenuation in the mouse model.

In light of the data presented above, we concluded that although VP1, VP2, VP5 and NS3 were major determinants of BTV8 virulence, other proteins also played a role in $BTV8_H$ attenuation, albeit to a lesser extent. As we had already rescued $BTV8_L+S1_H$, $BTV8_L+S2_H$, and $BTV8_L+S8_H$, our next step was to rescue the remaining monoreassortants with the $BTV8_L$ backbone. As previously, we assessed virulence of these viruses in $IFNAR^{-/-}$ mice. The majority of these reassortants caused clinical symptoms starting from 2 d.p.i. and 100% mortality in the first week of the experiment (Figure 20). In this experiment, $BTV8_L+S4_H$ and $BTV8_L+S10_H$ were the only viruses that showed some degree of attenuation in $IFNAR^{-/-}$ mice. In particular, $BTV8_L+S10_H$ did not cause any deaths when inoculated at 300 PFU and showed reduced mortality compared to $rgBTV8_L$ (40%) at the dose of 3000 PFU. $BTV8_L+S4_H$ caused 60% mortality and 100% mortality at 300 and 3000 PFU, respectively. These data confirmed the previous conclusion that S4 played a role in BTV8 pathogenicity. $S6_H$ and $S7_H$ however did not confer attenuation when present without other $BTV8_H$ segments.

To summarise, assessment of virulence of $BTV8_{L/H}$ reassortants in $IFNAR^{-/-}$ mice showed that virulence of BTV8 was a multigenic phenomenon and involved at least six determinants i.e. VP1, VP2, VP4, VP5, VP7 and NS3, in this experimental model. Figure 20 summarises mortality data obtained from individual experiments.

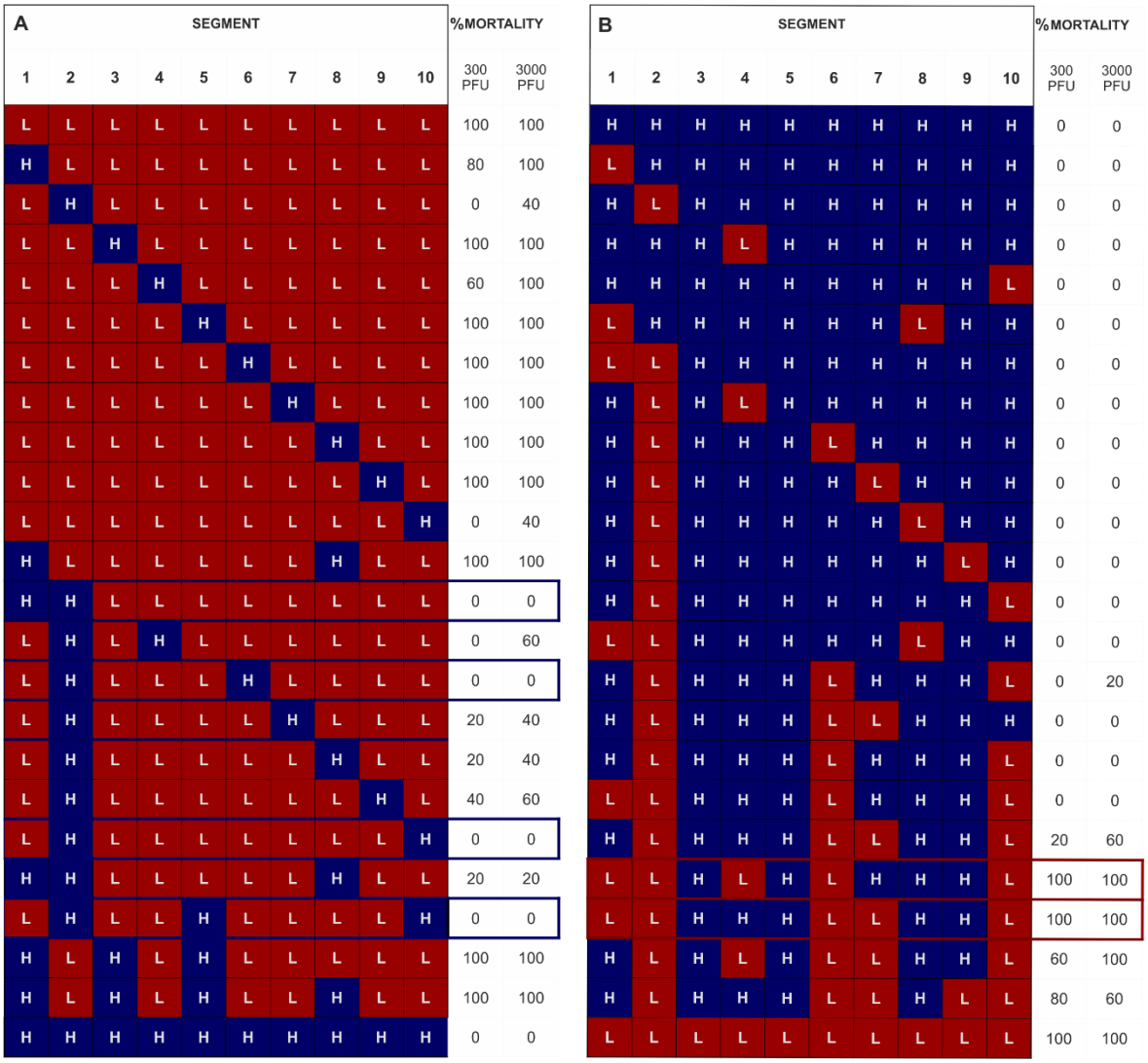

Figure 20. Mortality of IFNAR-/- mice inoculated with BTV8$_L$/BTV8$_H$ reassortants. Groups of five IFNAR$^{-/-}$ mice were innoculated intraperitoneally with either 300 or 3000 PFU of reassortants and monitored for 14 d.p.i. The figure is divided into two separate panels that show mortality of reassortants with the BTV8$_L$ backbone (A) or the BTV8$_H$ backbone (B).

4.2.6 Segment 2 increases BTV8$_H$ fitness in CPT-Tert cells.

Having found that replacement of specific segments of the BTV8$_L$ backbone with BTV8$_H$ equivalents resulted in the attenuation of the resulting reassortants *in vivo*, we then wanted to establish whether these segments also affected their replication *in vitro*. We assessed growth kinetics of a full set of 10 monoreassortants containing the BTV8$_L$ backbone in CPT-Tert cells, hence without IFN constraints, and compared their growth to parental viruses. Confluent monolayers were infected at a MOI of 0.01 with each monoreassortants and supernatant were collected 2, 24, 48 and 72 h.p.i. Additionally, we performed plaque assays in order to compare the sizes of plaques produced by parental viruses and individual reassortants. Overall, the majority of monoreassortants demonstrated similar replication kinetics to rgBTV8$_L$ (Figure 21A). BTV8$_L$+S4$_H$ and BTV8$_L$+S9$_H$ had slightly lower titres than rgBTV8$_L$ over the 72 h period but no substantial reduction in growth was shown by any of the viruses. Strikingly, BTV8$_L$+S2$_H$ demonstrated growth comparable with rgBTV8$_H$ and reached over 100 fold higher titres than rgBTV8$_L$ at 72 h.p.i. Moreover, rgBTV8$_H$ and BTV8$_L$+S2$_H$ produced larger plaques at 48 h.p.i. than rgBTV8$_L$ and the remaining monoreassortants (Figure 21B). We did not detect any significant variation in plaque sizes produced by the other assayed viruses. Overall, these data indicated that rgBTV8$_H$ replicated much better in CPT-Tert cells than rgBTV8$_L$ and that VP2 was the main determinant of this increased fitness in this particular cell line.

We also carried out several virus replication kinetic assays in order to assess whether the attenaution *in vivo* of some reassortants corresponded to an overall decreased replication fitness also *in vitro.* However, none of the attenuated reassortants showed significantly decreased yields compared to parental rgBTV8$_L$ (Figure 22).

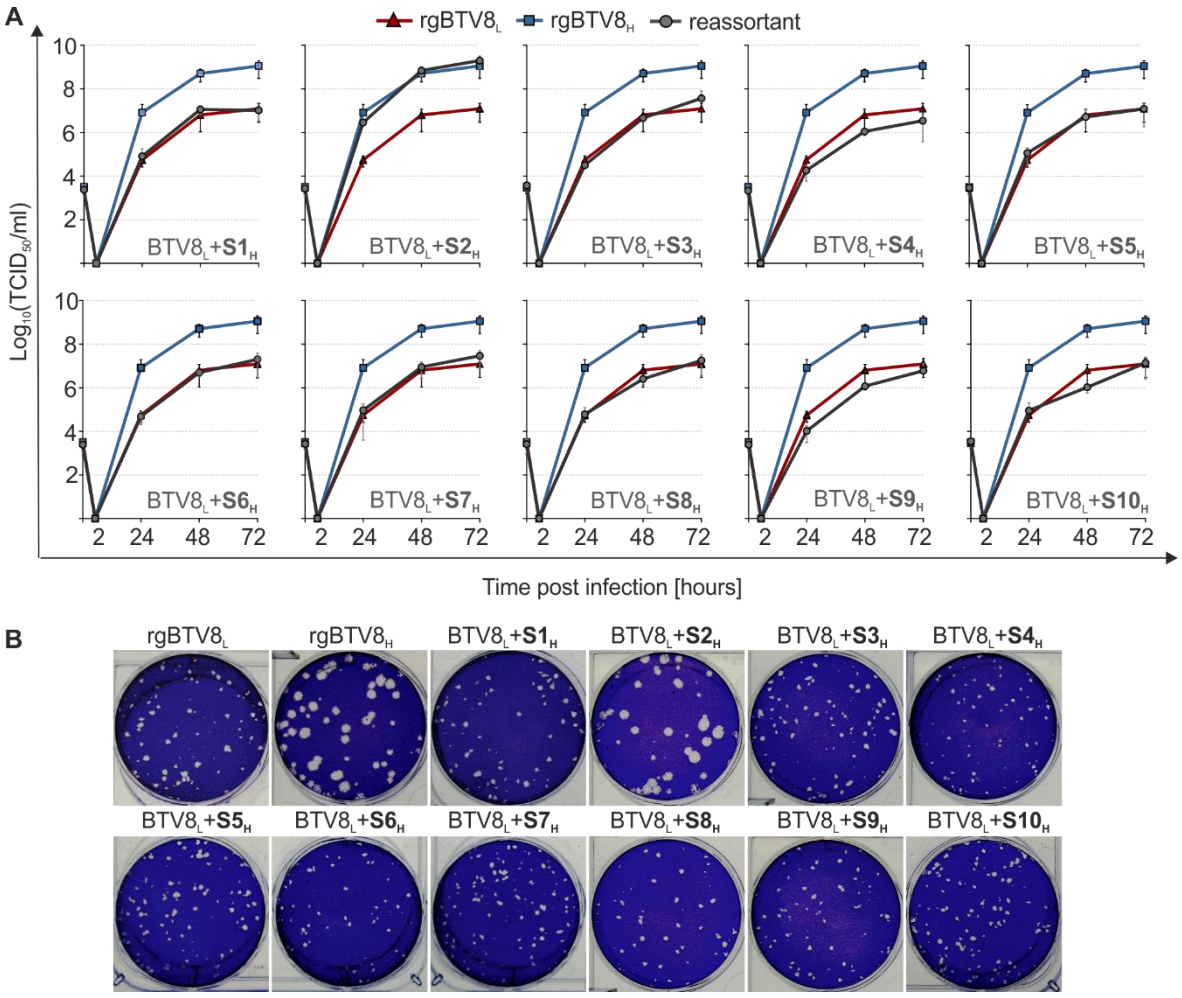

Figure 21. Replication kinetics of BTV8$_L$/BTV8$_H$ monoreassortants. (A) Growth curves of parental rgBTV8$_L$ (red triangle), rgBTV8$_H$ (blue square) and monoreassortants containing the BTV8$_L$ backbone (grey circle) in CPT-Tert cells. Monolayers were infected with the indicated viruses at MOI 0.01 and supernatants collected at 2, 24, 48 and 72 h p.i. Viral titres were determined by endpoint dilutions. Growth curves were performed three times in duplicate; error bars correspond to standard deviation. All reassortants, with exception of BTV8$_L$+S2$_H$, showed replication kinetics similar to parental rgBTV8$_L$. (B) Plaques produced in CPT-Tert cells by parental rgBTV8$_L$ and rgBTV8$_H$ and derived monoreassortants 48h p.i. Note the increased plaque size in rgBTV8$_H$ and BTV8$_L$+S2$_H$.

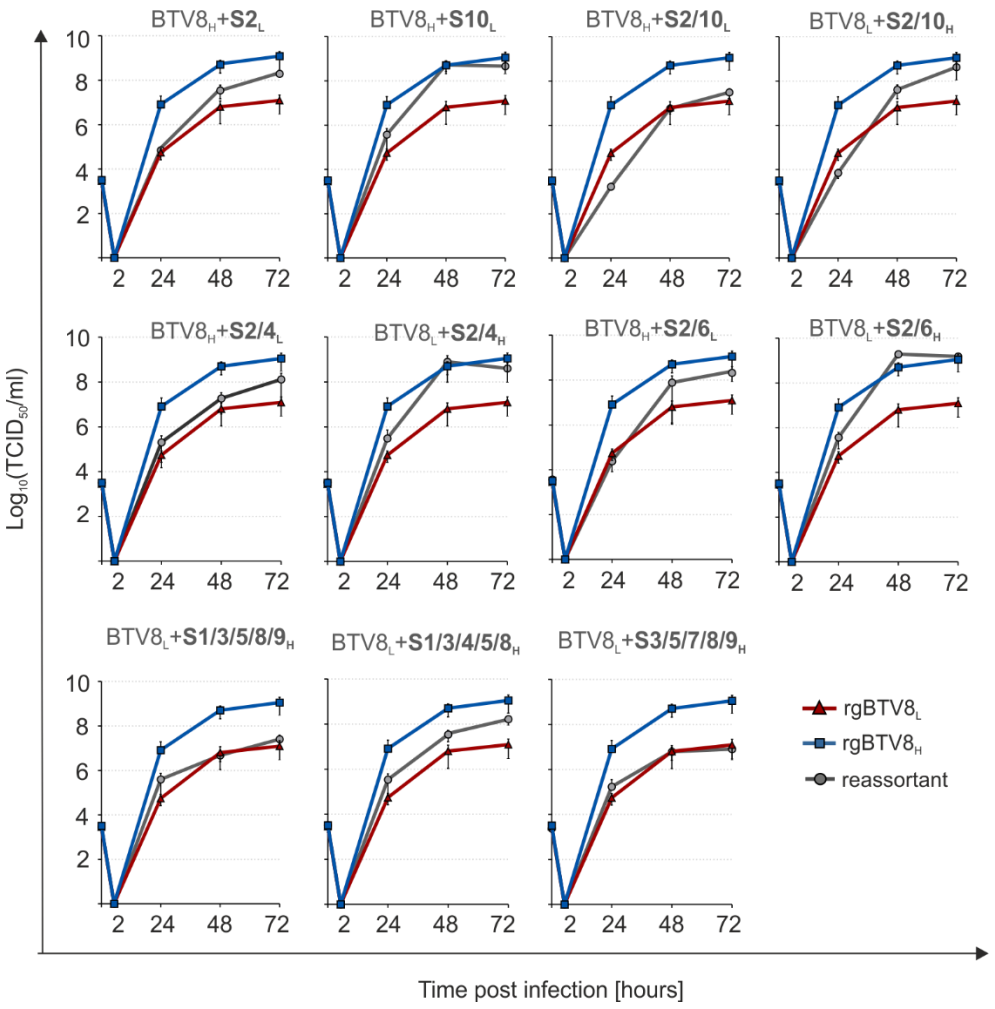

Figure 22. Replication kinetics of BTV8$_L$/BTV8$_H$ reassortants. Growth curves of parental rgBTV8$_L$ (red triangle), rgBTV8$_H$ (blue square) and monoreassortants containing the BTV8$_L$ backbone (grey circle) in CPT-Tert cells. Monolayers were infected with indicated viruses at MOI 0.01 and supernatants collected at 2, 24, 48 and 72 h p.i. Viral titres were determined by endpoint dilutions. All reassortants showed replication kinetics similar or better than parental rgBTV8$_L$. Error bars corresponding to standard deviations are shown.

4.2.7 $S2_H$ confers increased affinity for glycosaminoglycans *in vitro.*

VP2 is the main determinant of BTV serotype and mediates viral attachment and cell entry (Forzan et al., 2007; Hassan and Roy, 1999; Huismans and Erasmus, 1981; Huismans et al., 1987a; Mertens et al., 1989). Cells cultured *in vitro* tend to increase expression of glycosaminoglycans (GAGs) at the cell membrane. Interestingly, some viruses like foot and mouth disease virus (FMDV) (Baranowski et al., 1998) show an increase in affinity for heparan sulphate after passaging *in vitro*. Since our data showed that $rgBTV8_H$ produced much higher yields in CPT-Tert cells than $rgBTV8_L$, we hypothesised that extensive passage in tissue culture affected $rgBTV8_H$ binding to GAGs. Hence, we performed viral replication kinetic assays in CHO cells expressing GAGs ubiquitously and in a derived cell line, CHO-pgsA745, deficient in xylotransferase and lacking therefore heparan sulphate glycosaminoglycans. $RgBTV8_L$, or an $rgBTV8_H$ reassortant with the VP2 of $BTV8_L$ ($BTV8_H$+$S2_L$), grew equally well in both cells lines. However, $BTV8_H$ and $BTV8_L$+$S2_H$ reached approximately 10 folds higher titres in CHO cells (Figure 23). This confirmed that the VP2 of $BTV8_H$ had a higher affinity for GAGs thus facilitating BTV8 replication *in vitro*, but not *in vivo*.

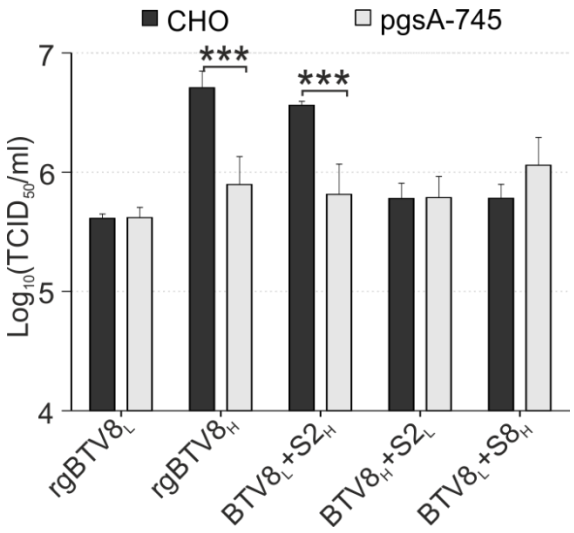

Figure 23. Viral titres reached in CHO and pgsA-745 cells infected by $rgBTV8_L$, $rgBTV8_H$ and reassortant viruses (MOI= 0.01). Supernatants were collected 72 h p.i. and titrated in BSR cells by limiting dilution analysis. Mean values from three experiments performed in duplicate are shown (error bars correspond to standard deviations). Note that significant differences were observed between titres produced in CHO and pgsA-745 by $rgBTV8_L$+$S2_H$ and $rgBTV8_H$+$S2_L$ (*** = $p<0.001$; 2-way ANOVA followed by Bonferroni post-hoc test).

4.2.8 Poor replication of rgBTV8$_H$ in OvEC is not related to levels of expressed type 1 interferon.

In vitro experiments described in Chapter 3, showed that BTV8$_H$ was not able to replicate in IFN competent ovine cells suggesting that the IFN response could be crucial in attenuation of the high passage virus in its natural host. Thus, we further explored this model to characterise differences between rgBTV8$_L$ and rgBTV8$_H$ interactions with the cellular IFN system, as well as the involvement of specific segments in these interactions. First, we used parental viruses and the set of single-segment reassortants and compared their replication kinetics in OvEC infected at MOI of 0.01. Interestingly, in these cells we found a spectrum of different growth patterns produced by various monoreassortants (Figure 24). BTV8$_L$+S2$_H$ displayed a replication efficiency superior to the other tested viruses, which suggested that mutations in S2$_H$ conferred an advantage to *in vitro* growth irrespective to cells ability to produce IFN. Several reassortants demonstrated delayed growth compared to rgBTV8$_L$. In particular, BTV8$_L$+S4$_H$ and BTV8$_L$+9$_H$ produced substantially lower titres than the parental virus at 48 and 72 h.p.i. None of the reassortants however, replicated as poorly as rgBTV8$_H$, which indicated that the growth restriction of high passage BTV8 was a cumulative result of mutations in several genome segments.

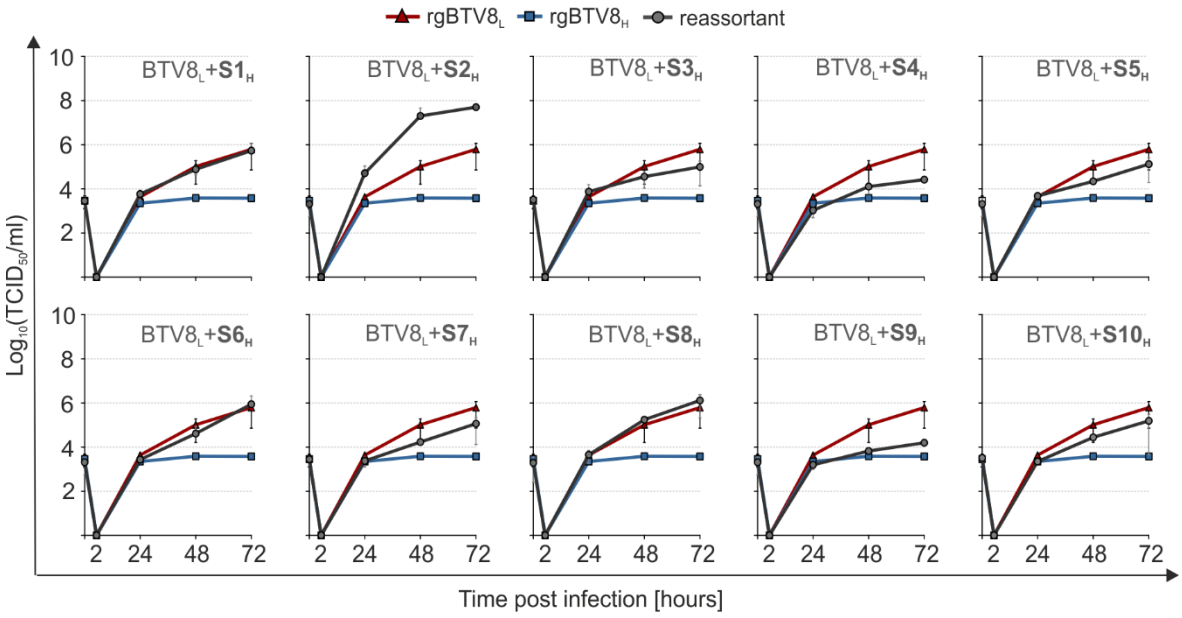

Figure 24. Replication kinetics of parental and monoreassortant viruses in primary OvEC cells. OvEC cells were infected at MOI 0.01 with rgBTV8$_L$ (red triangle), rgBTV8$_H$ (blue square) and monoreassortants containing BTV8$_L$ backbone (grey circle). Supernatant samples were collected at 2, 24, 48 and 72 h.p.i. and viral titres were determined at the specified time points by limiting dilution assays. Each panel shows growth curves of both parental viruses and a specific monoreassortant (as labelled). The experiment was performed twice in duplicate and error bars correspond to standard deviations.

Next, we wanted to establish whether through extensive passage with no IFN constraints, $BTV8_H$ lost the ability to counteract the IFN production in infected cells and became a more potent IFN inducer than the original $BTV8_L$. To this end, we assayed IFN production in OvEC cells infected with the same set of rescued viruses at MOI 1 and measured the levels of IFN in the supernatants collected 18 hours later. Surprisingly, we did not find any significant difference between IFN levels produced by OvEC infected with the two parental viruses. Moreover, most of the reassortants induced similar amounts of IFN (Figure 25A). A statistically significant difference ($p< 0.05$) was observed between $rgBTV8_L$ and $BTV8_L+S9_H$. Interestingly the latter induced approximately six times less IFN in OvEC than the parental virus, which could be a result of less efficient replication in these cells. No IFN was detected in the mock-infected samples.

To confirm our results further, we measured the relative quantities of the IFN-β gene and selected ISGs mRNA (MX1 and RSAD2) and β-actin in OvEC cells infected with $rgBTV8_H$, $rgBTV8_L$ and the various monoreassortants (MOI = 1) and collected at 18 h.p.i. In addition to infections with rescued viruses, mock infected and UIFN treated cells were used as controls. We detected no IFN-β RNA in either of the control samples while it was readily detectable at similar levels in cells infected with $rgBTV8_L$, $rgBTV8_H$ and the $rgBTV8_L$/$rgBTV8_H$ monoreassortants (Figure 25B). No significant differences were found in the expression of IFN-β in cells infected with different viruses. Similarly, there was a marked induction of RSAD2 and MX1 in all virus-infected samples compared to mock-infected controls. However, no significant variation was found in expression of the ISG in cells infected with the various monoreassortants and the parental viruses. Act-β levels were consistently uniform in all samples. Together, these data demonstrated that inhibition of $rgBTV8_H$ replication in OvEC and reduced growth of selected monoreassortants was not related to the levels of IFN produced in infected cells.

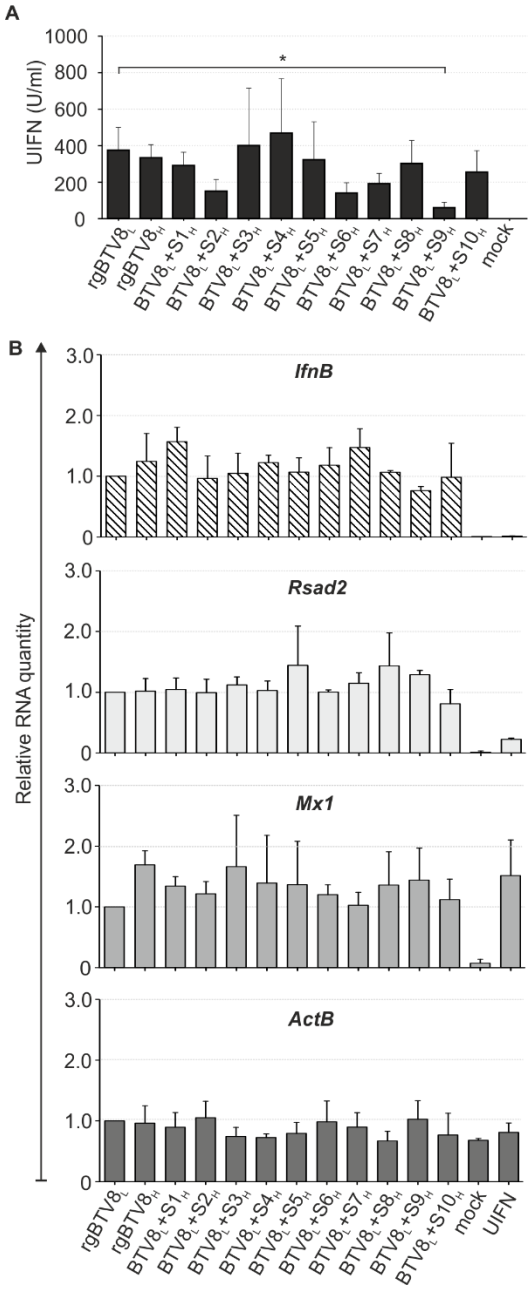

Figure 25. IFN production and gene expression induced by infection of OvEC by rgBTV8$_L$, rgBTV8$_H$ and BTV8$_L$/BTV8$_H$ monoreassortants. OvEC cells were infected with rgBTV8$_L$, rgBTV8$_H$ and monoreassortants within the BTV8$_L$ backbone (MOI=1). (A) IFN protection assays. Supernatants were collected at 18h p.i., inactivated by UV treatment and used in a biological assay to estimate the amount of IFN present as described in Materials and Methods. The only major differences were observed in cells infected with BTV8$_L$+S9$_H$ where the amount of IFN released was significantly lower than what was found in cells infected with rgBTV8$_L$ ($p<0.05$; 1 way ANOVA followed by Dunnett's multiple comparison test to dissect individual interactions). (B) *IFNB, ActB, Rsad2 and Mx1* expression. mRNA was measured by qPCR in OvEC 18h p.i. with parental and reassortant viruses (MOI = 1) as described in Materials and Methods. Mock-treated and UIFN-treated cells were used as controls. Panels show gene expression relative to rgBTV8$_L$ and normalised to *GAPDH* levels. Error bars correspond to standard deviations.

4.2.9 Mutations in multiple segments reduce rgBTV8$_H$ capability to replicate in IFN pre-treated CPT-Tert cells.

The data illustrated above, showed that the reduced replication kinetics of rgBTV8$_H$, BTV8$_L$+S4$_H$ and BTV8$_L$+S9$_H$ in OvEC were not due to an increased IFN induction in these cells. In light of these data, we wanted to establish whether dramatically inhibited growth of rgBTV8$_H$ in an IFN competent cell line was due to its reduced ability to overcome restriction factors in cells activated by IFN prior to infection. For these experiments, we used CPT-Tert cells that do not produce IFN but will respond to exogenous IFN (Arnaud et al., 2010; Ruscanu et al., 2012; Varela et al., 2013) We pre-treated cells with 1000 units of UIFN or control media for 18 h prior to infection with rgBTV8$_L$, rgBTV8$_H$ or monoreassortants with a BTV8$_L$ backbone at MOI 0.01. In parallel, we infected untreated cells with the same set of viruses and 48 h.p.i. we collected supernatants from IFN-treated and untreated samples. Supernatants were used to determine viral titres. Cells were stained with crystal violet to visualise CPE 72 h.p.i. Comparison of viral yields in cells treated and untreated with UIFN showed that replication of all viruses was significantly inhibited by UIFN and titres in untreated cells were more than 100-fold higher than in the UIFN pre-treated samples (Figure 26A). The reduction of rgBTV8$_L$ yield in UIFN-treated cells, compared to untreated cells, was approximately $5x10^3$-fold. Strikingly, this ratio was more than a million fold ($1.7x10^6$) for rgBTV8$_H$. Moreover, the titre of rgBTV8$_H$ in UIFN-treated cells at 48 h.p.i. was 16 times lower than the titre of rgBTV8$_L$. Most reassortants showed similar yields in UIFN-treated cells as the parental low passage virus. A notable exception was BTV8$_L$+S4$_H$, which reached tenfold lower titres than rgBTV8$_L$ ($p < 0.05$) under these conditions. BTV8L+S2$_H$ showed the highest degree of inhibition in treated CPT-Tert cells among all the ten monoreassortants. However, the yield of BTV8L+S2$_H$ in treated cells was equivalent to the one obtained by rgBTV8$_L$ in the same conditions. This suggested that the increased efficiency of replication conferred by VP2$_H$ could be limited to a large extent in cells that were activated by IFN prior the infection.

Plaque comparison in UIFN pre-treated cells showed that while most of the viruses caused visible CPE, no detectable plaques were formed in rgBTV8$_H$-infected cells (Figure 26B). BTV8L+VP4$_H$ showed decreased CPE compared to rgBTV8$_L$ but this reduction was not as dramatic as in the case of rgBTV8$_H$. Collectively, these data demonstrated that

rgBTV8$_H$ was not able to overcome the restriction barriers in cells already in an antiviral state and its replication was limited due to mutations present in multiple segments including VP4.

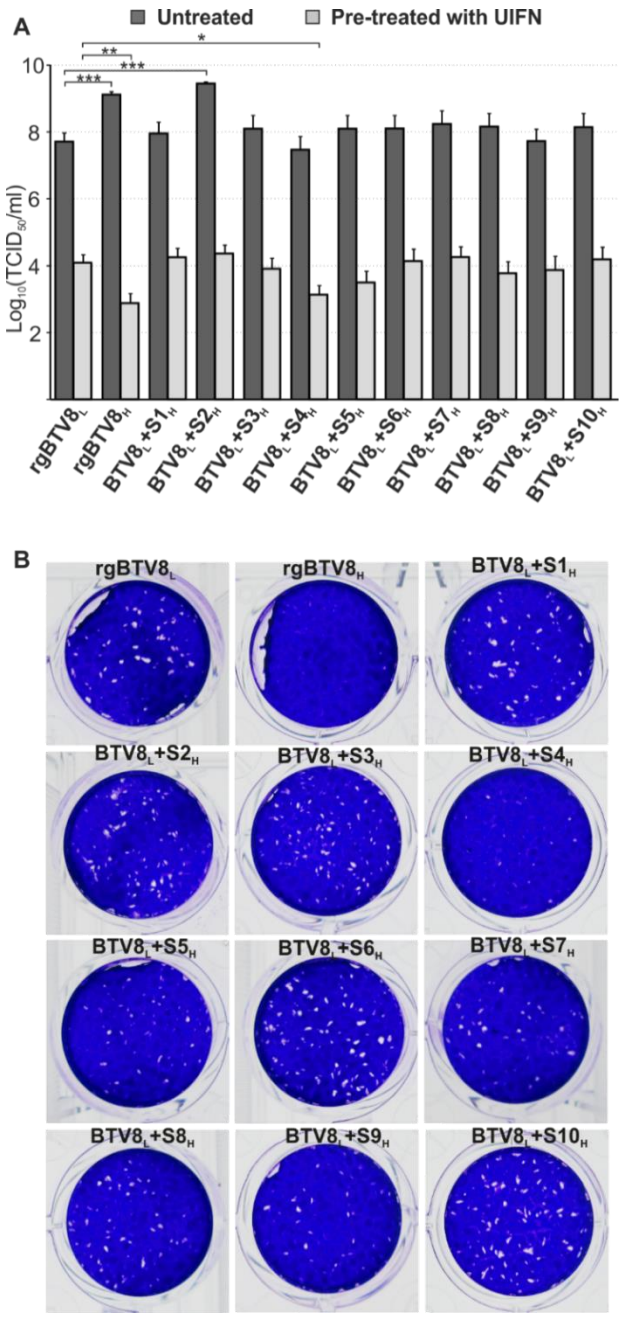

Figure 26. Replication of rgBTV8$_L$, rgBTV8$_H$ and BTV-8$_L$/BTV-8$_H$ monoreassortants in CPT-Tert cells pre-treated with universal IFN (UIFN). (A) Viral titres produced in untreated (dark grey) and IFN-pretreated (light grey) CPT-Tert cells by parental and reassortant viruses 48 h.p.i. at MOI 0.01. Mean values from three experiments performed in duplicate are shown (error bars correspond to standard deviations; *= $p < 0.05$; ** = $p< 0.01$; ***= $p< 0.001$; 1 way ANOVA followed by Dunnett's multiple comparison test to dissect individual interactions). (B) Plaques produced in CPT-Tert cells pretreated with 1000U/ml of UIFN by parental rgBTV8$_L$ and rgBTV8$_H$ and derived monoreassortants 72 h.p.i. Note that no plaques were formed by rgBTV8$_H$ and decreased size plaques by BTV8$_L$+S4$_H$.

4.2.10 RgBTV8$_H$ infection leads to increased activation of IFN-β promoter in A549 cells.

A549 are cells of human origin that have been used previously to study BTV interactions with the innate immune system (Chauveau et al., 2012; Doceul et al., 2014). The cells are able to produce and respond to IFN and therefore could potentially be used to discern the differences between BTV8$_L$ and BTV8$_H$ in the interactions with the IFN system. In the assays we performed previously in OvEC cells, we found no differences in expression of the IFN-β gene nor production of IFN in cells infected with rgBTV8$_H$, rgBTV8$_L$ or individual reassortants. We therefore wanted to check whether this could be confirmed also in cell lines of human origin. Hence, we have obtained A549/pr(IFN-β).GFP reporter cells that express GFP under the control of the IFN-β promoter (Chen et al., 2010) and are therefore useful for the analysis of IFN induction in virus-infected samples.

We infected A549/pr(IFN-β).GFP cells with rgBTV8$_H$, rgBTV8$_L$ or monoreassortants (MOI 1.5). 48 h.p.i. cells were trypsinised and fixed with formaldehyde. Strikingly, we found a major difference between the amounts of GFP-positive cells in rgBTV8$_H$ or rgBTV8$_L$ samples (Figure 27). Infection with rgBTV8$_L$ did not induce considerable GFP expression, and only a few green fluorescent cells were detected. RgBTV8$_H$-infected cells on the contrary showed high GFP expression. With the exception of BTV8$_L$+S4$_H$, infection with reassortant viruses did not cause an increased GFP production. We used FACS to quantify the percentage of green fluorescent cells in our samples. We detected approximately six times more GFP positive cells in rgBTV8$_H$-infected cells compared with rgBTV8$_L$ samples (9.3% and 1.5% respectively). No statistically significant differences were found in the numbers of GFP$^+$ cells between cultures infected with rgBTV8$_L$ and those infected with the majority of monoreassortants. The exception was BTV8$_L$+S4$_H$ which induced significantly more GFP than the parental low passage virus ($p<0.01$). However, the mean number of fluorescent cells was almost two times smaller than in rgBTV8$_H$ samples (5.1% for BTV8$_L$+S4$_H$). Altogether, these data showed that in contrastto what was observed in OvEC, in A549 cells rgBTV8$_H$ had a reduced ability to down-regulate the IFN response directly or was sensed more efficiently by the infected cells, which led to a more potent IFN promoter activation.

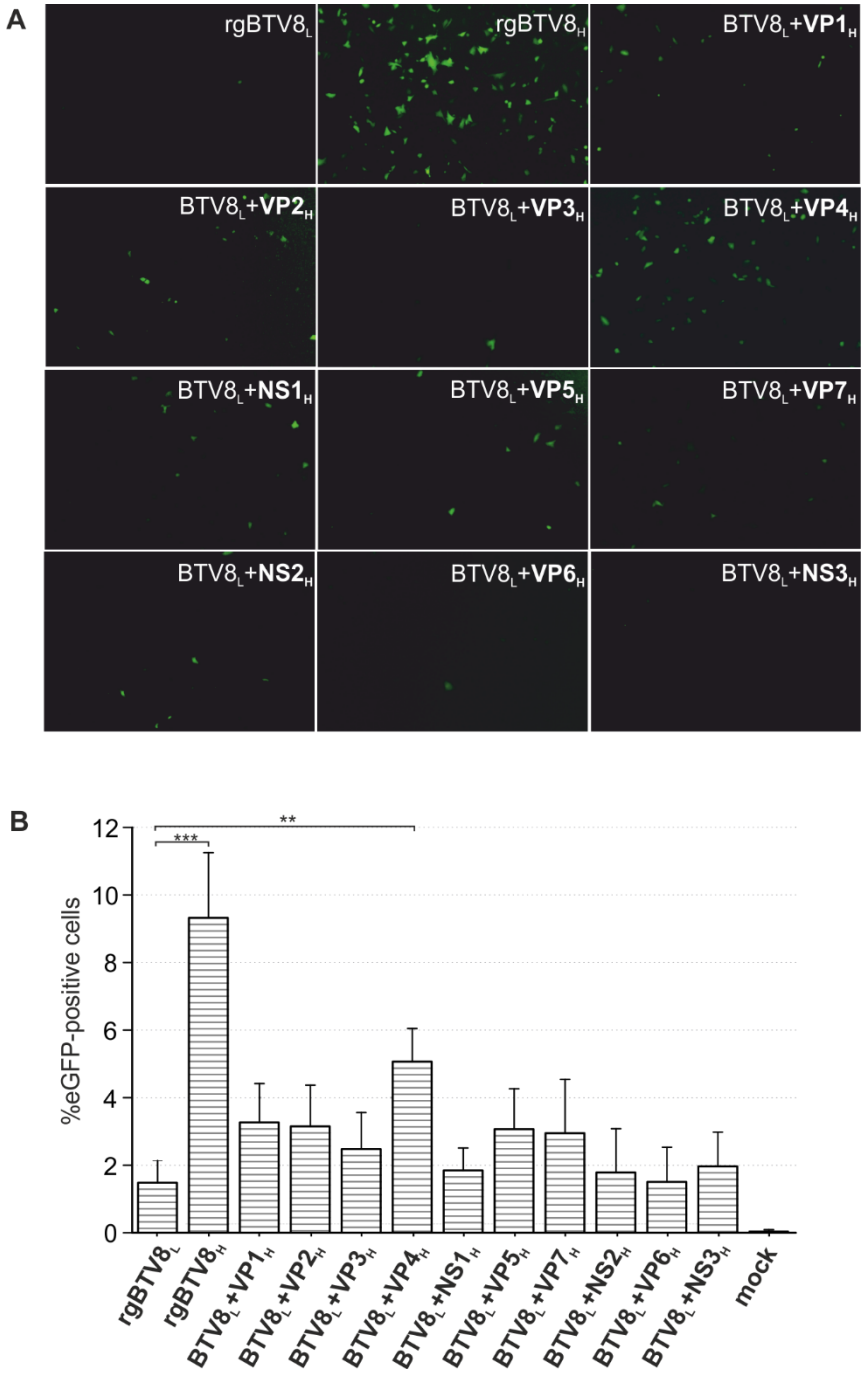

Figure 27. Upregulation of IFN-β expression in cells infected with rgBTV8$_L$, rgBTV8$_H$ and monoreassortants. (A) GFP fluorescence in A549/pr(IFN-β).GFP infected with rgBTV8$_L$, rgBTV8$_H$ or specified monoreassortants (MOI 1.5). (B) Proportion of GFP fluorescent cells in A549/pr(IFN-β).GFP infected with MOI 1.5 of indicated viruses. The cells were harvested and formalin-fixed 48 h.p.i. and the number GFP positive cells in each sample was counted by FACS as described in Materials and Methods. Mean values from three experiments performed in duplicate are shown (error bars correspond to standard deviations; ** = p< 0.01; ***= p< 0.001; 1-way ANOVA followed by Dunnett's multiple comparison test to dissect individual interactions).

To assess whether increased prIFN activation resulted in inhibition of rgBTV8$_H$ replication we titrated the parental viruses and monoreassortants in A549 cells. Confluent monolayers were infected with MOI 0.01 of selected viruses and supernatants were collected at 2, 24, 48 and 72 h.p.i. Surprisingly, rgBTV8$_H$ gave higher yields than rgBTV8$_L$. We noted almost 1 log difference in titres between both parental viruses at 72 h.p.i. Growth of BTV8$_L$+S2$_H$ closely mirrored that of the high passage virus which confirmed our previous conclusion that VP2 mediated increased replication efficiency of rgBTV8$_H$ *in vivo*. Curiously, unlike in primary ovine cells, replication of high passage virus was not inhibited in A549 cells. However, BTV8$_L$+S4$_H$ and BTV8$_L$+S9$_H$ failed to grow as efficiently as rgBTV8$_L$ in both OvEC and in A549 cells. Both these reassortants reached approximately 10 times lower titres than the parental low passage virus at 72 h.p.i. BTV8$_L$+S7$_H$ showed intermediate growth efficiency when compared with parental viruses, which suggested that VP7$_H$ was advantageous over the BTV8$_L$ equivalent protein in this cell line.

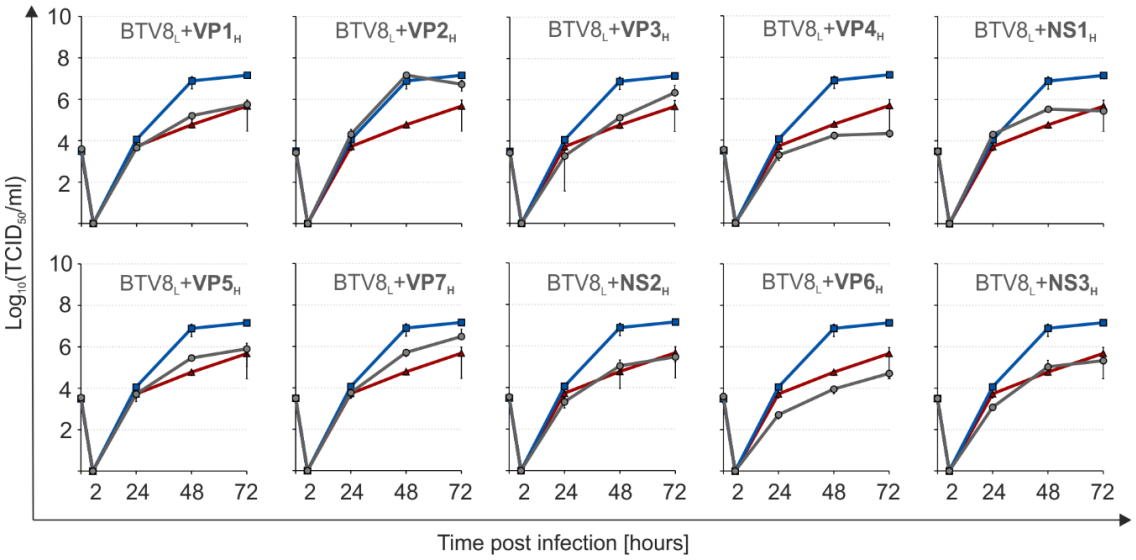

Figure 28. Growth kinetics of rgBTV8$_L$, rgBTV8$_H$ and monoreassortants in A549 cells. A549 cells were infected at MOI 0.01 with rgBTV8$_L$ (red triangle), rgBTV8$_H$ (blue square) and monoreassortants containing BTV8$_L$ backbone (grey circle). Supernatant samples were collected at 2, 24, 48 and 72 h.p.i. and viral titres were determined at the specified time points by limiting dilution assays. Experiment was performed twice in duplicate and error bars correspond to standard deviation values.

4.3 Discussion

In this chapter, we identified the molecular determinants of pathogenicity of a virulent strain of BTV-8. We rescued a set of reassortant viruses between $BTV8_H$ and $BTV8_L$ and described their phenotypes *in vitro* and *in vivo*. Our approach allowed us to identify specific genetic differences that occurred as a consequence of virus passage in an IFN-defective cell line, in order to understand the involvement of individual genomic segments in BTV-8 adaptation to tissue culture and attenuation *in vivo*.

Here, we found that mutations in four segments (S1, S2, S6, S10) encoding VP1, VP2, VP5 and NS3 contributed to attenuation of $BTV8_H$ in $IFNAR^{-/-}$ mice. In particular, reassortants with the $rgBTV8_L$ backbone and VP2 or NS3 from $rgBTV8_H$ caused no mortality when inoculated at 300 PFU dose. These data are an accord with the study of Caporale *et al.* who showed that VP1 and VP2 were two of three proteins consistently mutated in tissue culture adapted strains derived from Italian strains of BTV-2, BTV-4 and BTV-9 (Caporale et al., 2011). $S2_H$ with either $S1_H$, $S6_H$ or $S10_H$ in the context of the $rgBTV8_L$ backbone fully attenuated the resultant reassortants but, surprisingly, replacing these four proteins with the equivalents of $rgBTV8_L$ in the $rgBTV8_H$ backbone did not restore virulence of the latter. The fact that partial virulence was achieved only after combining $VP7_L$ with $VP2_L$, $VP5_L$ and $NS3_L$ strongly suggests that interactions between specific viral proteins could play a role in the pathogenicity of BTV. Both VP2 and VP5 have previously been shown to interact with VP7 trimers in the BTV particle (Nason et al., 2004). Additionally, other studies demonstrated the importance of NS3 interactions with outer capsid proteins in virus trafficking, assembly and inter-serotype pathogenicity in the natural host (Beaton et al., 2002; Bhattacharya and Roy, 2008; Celma et al., 2014). It is therefore possible that while mutations in high passage VP5 or VP7 did not directly affect functions of these proteins, they did influence the pathogenicity through more compatible interactions with VP2 and NS3 derived from the same strain.

Interestingly, two mutations found in VP2 of $BTV8_H$ (positions 321 and 328) were located at the same region that was previously associated with attenuated BTV strains and identified as a target for neutralising antibodies (Gould and Eaton, 1990). This external and highly exposed area of VP2 was also implicated in attachment to a host cell receptor and the mutations that arise in this region could be due to the changes in affinity for

binding to specific ligands (Zhang et al., 2010). The receptor binding protein of BTV, VP2, has been well characterised but only a few studies focused on identifying its cellular target. Cryoelectron microscopy studies showed that VP2 possess a sialic acid binding region located in its hub domain, which is one of two sites suggested to be interacting with the cell surface receptor (Zhang et al., 2010). Additionally, VP2 alone is responsible for agglutination of ruminant erythrocytes through glycophorins, which further confirms sialic binding properties of VP2 (Eaton and Crameri, 1989; Hassan and Roy, 1999). The presence of another putative receptor-binding site at the VP2 tip domain however strongly suggest that BTV utilizes another cellular factor for cell entry. Moreover, wheat germ competition assay studies showed that in the presence of wheat germ protein, which blocks sialic acid sites on the cell surface, BTV infectivity was reduced but not entirely abolished (Zhang et al., 2010). Altogether, these data show that BTV entry is facilitated by sialic acid but it also requires another unidentified receptor. It is common for many viruses to utilise more than one cell surface factor for attachment. The primary interaction with the host cell might first occur via non-specific molecules followed by high affinity binding to another host receptor. In particular, an increased affinity for binding to glycosaminoglycans (GAGs) has often been cited in the context of tissue culture adapted strains (Baranowski et al., 1998; Gardner et al., 2014; Klimstra et al., 1998; Mandl et al., 2001). Here we found that $BTV8_H$, or reassortants with the VP2 of $BTV8_H$, had indeed a greater affinity for GAGs and reached titres approximately 10 fold higher in wild type CHO cells compared to cells lacking GAGs. Previous studies have shown that viruses that acquire mutations that confer the ability for attachment to GAGs, and in particular to heparan sulphate proteoglycans, are often attenuated *in vivo* (Bernard et al., 2000; Byrnes and Griffin, 2000; Lee et al., 2006; Mandl et al., 2001; Olmsted et al., 1984). Similarly, $BTV8_L+S2_H$ was attenuated in $IFNAR^{-/-}$ mice despite the fact that in tissue culture (both in IFN deficient and competent cells) it replicated to higher titres than any other monoreassortant. Studies looking into dissemination of S2 reassortants in low or high passage backbone would be necessary to understand whether increased binding to GAGs leads to sequestration of the virus at sites not favourable for replication and/or to a more efficient clearance of the virus from blood. Moreover, the $BTV8_H$ VP2 acquired four amino acid mutations during passage in BSR cells. Consequently, to confirm which mutation was responsible for the change in receptor affinity it will be necessary to rescue reassortant viruses that contain individual

mutations in S2 separately. Binding to GAGs occurs through interactions with positively charged amino acids of the viral receptor binding protein. Therefore, increased affinity for GAG attachment is acquired through amino acid mutations into His, Lys or Arg residues (Byrnes and Griffin, 1998). Only one mutation in S2 (Glu to Lys substitution at position 16) fit this description and hence it is most likely that this mutation is responsible for increased binding to cell surface GAGs.

BTV is a potent inducer of type 1 interferon *in vivo* and *in vitro* and the ability of the virus to counteract the host IFN response was linked to its pathogenicity (Chauveau et al., 2013; Huismans, 1969; Jameson et al., 1978; Maclachlan and Thompson, 1985; Ratinier et al. 2011; Ruscanu et al. 2012). The IFNAR$^{-/-}$ mouse is a well-established model of bluetongue and due to the lack of expression of alpha/beta IFN receptor, it is a suitable tool to study the determinants of pathogenicity that are unrelated to IFN expression (Calvo-Pinilla et al., 2010; Caporale et al., 2011; Ortego et al., 2014). Using this mouse model, we demonstrated the involvement of several segments in the pathogenicity of BTV-8. We cannot exclude however that other factors, IFN system related, additionally contributed to decrease pathogenicity of rgBTV8$_H$ in the natural host. As studies on the pathogenicity of multiple reassortants in sheep are not feasible, we used primary OvEC cells as an *in vitro* "surrogate" model to demonstrate the involvement of IFN in restriction of the growth BTV8$_H$ and its reassortants. Recently, two BTV proteins have been implicated in counteracting the host IFN system (Chauveau et al., 2013; Ratinier et al., 2011; Vitour et al., 2014). In particular, NS3 has been shown to interfere with the IFN synthesis in mammalian cells (Chauveau et al., 2013). However, we saw no significant variation in the IFN production in OvEC infected with parental viruses or single segment reassortants while their growth patterns in the same cell line differed. Most of the reassortants were able to replicate to similar titres as the low passage virus. Two notable exceptions included BTV8$_L$+S4$_H$ and BTV8$_L$+S6$_H$. It is however essential to remark that VP4 also influenced viral phenotype in IFNAR$^{-/-}$ mice, which would suggest that mutations in this protein could also have affected its functions unrelated to the IFNAR signalling cascade. The VP4 of BTV acts as a capping enzyme and therefore mutations in S4$_H$ could have an adverse effect on the efficiency of viral mRNA capping (Ramadevi and Roy, 1998; Sutton et al., 2007). A recent study of Stewart *et al.* highlighted the importance of the K-D-K-E amino acid tetrad and the surrounding residues in the efficiency of BTV 2'OMTase

and the effect of its mutation on the virus fitness (Stewart and Roy, 2015). Importantly, mutations in some of these residues led to decreased activity of guanyltransferase and showed a slight delay in replication assays, compared to wild type virus (Stewart and Roy, 2015). Moreover, recent studies showed that viral mRNA lacking 2′-O-methylation at their 5′ cap structure induces more potent innate immune response through Mda5 activation or direct interactions with proteins from the IFIT family (Daffis et al., 2010; Garcia-Sastre, 2011; Zust et al., 2013). Inefficient capping mechanism would therefore explain the slight, yet consistent decrease in virus yields reached by BTV8L+$S4_H$ in IFN deficient cell lines. This hypothesis was further supported by comparing growth kinetics of monoreassortants in the $BTV8_L$ backbone in CPT-Tert cell line untreated or pre-treated with UIFN. It has been shown that pre-treatment of cells with UIFN leads to initial decrease in BTV growth efficiency but with time, the virus is able to overcome this restriction (Ratinier et al., 2011). Indeed, we saw that growth of $BTV8_L$ and most reassortants was inhibited by UIFN approximately 1000 fold. Replication of rg$BTV8_H$ on the other hand was inhibited more than a million fold in cells in an antiviral state. Of all reassortants, BTV8L+$S4_H$ showed the lowest titres 48 h after UIFN pre-treatment which suggested that $VP4_H$ was one of the proteins contributing to the inability of rg$BTV8_H$ to replicate in cells primed with IFN. These data are in concordance with the study of Daffis and colleagues who demonstrated that West Nile Viruses, Coronaviruses and Poxviruses with deficient 2'OMTase activity were not able to escape IFIT-2 induced restriction in transgenic cells stably expressing IFIT-2 (Daffis et al., 2010). It is therefore possible that through viral mRNA capping, the VP4 of BTV-8 could play a role in evading host restriction factors to allow the virus to replicate in host cells already induced in an antiviral state.

VP6 is encoded by S9 of BTV genome, as is the NS4 protein, which is the second of the BTV proteins shown to counteract the IFN system (Ratinier et al., 2011). However, we found no mutations in the NS4 open reading frame but we detected a non-synonymous mutation in VP6. There is no evidence in the literature of VP6 or other viral helicases being involved in interactions with the innate immune system. It is therefore likely that the reduced growth of $BTV8_L$+$S6_H$ in OvEC was due to decreased replication efficiency in endothelial cells *per se*. We have noticed a minor decrease in yields $BTV8_L$+$S6_H$ in CPT-Tert cells (compared with $BTV8_L$) and the lowered replication efficiency could have been more evident in the OvEC. Additionally, expression of the IFN could have slowed the

replication rates of this virus even further. It is possible that specific interactions between minor proteins in the core (VP1, VP4 and VP6) rely on their structural compatibilities and VP6 of $BTV8_H$ was not fully efficient in the context of the $BTV8_L$ backbone.

It is important to stress that although the single segment reassortants displayed an array of intermediate growth patterns in OvEC, none of them replicated in these primary cells as poorly as $rgBTV8_H$. This indicated that mutations in several segments contributed to the restricted replication of $rgBTV8_H$. A similar study by Pérez-Cidoncha *et al.* demonstrated that multiple passage of Influenza virus in an IFN unresponsive cell line led to the emergence of viruses that were unable to counteract the effects of exogenous IFN (Perez-Cidoncha et al., 2014). Most of these viruses were shown to have mutations in proteins other than the immunomodulatory NS1 and several were identified as IFN hyper-inducers. Similarly, we found no mutations in the NS4 protein and the single amino acid substitution in NS3 did not affect the amount of IFN induced in response to $rgBTV8_H$ infection in OvEC. Altogether, these data show that passaging the virus with no constraints from the IFN system allows for greater flexibility of the entire genome, which in turn allows the emergence of viruses with optimal replication efficiencies. The mutations that arise in such conditions might not necessarily involve major IFN antagonists but can involve proteins that are normally fine-tuned to evoke minimal immune response while allowing sufficient (yet suboptimal) transmission in the natural host (Perez-Cidoncha et al., 2014). In the case of $BTV8_H$, the extensive passage in BSR cells led to the emergence of a virus with mutations in all 10 segments that likely complemented each other to form a virus with supreme replication efficacy in the system lacking immune defences. However, when the IFN was re-introduced into this system, some of these mutations became disadvantageous to the virus.

The results of *in vitro* experiments discussed above are derived from work using sheep cell lines. Several studies used a human cell line (A549) as a more convenient interferon competent infection model. Chauveau *et al*. demonstrated that this cell line expresses IFN-β and pro-inflammatory cytokines in response to BTV infection (Chauveau et al., 2012). We have therefore used A549 cells expressing GFP under the control of the interferon promoter (prIFN) to quantify the number of cells where prIFN was activated in response to either parental or monoreassortant viruses. Unexpectedly, we found that more cells expressed GFP after high MOI infection with $rgBTV8_H$ than $rgBTV8_L$.

Interestingly, $BTV8_L$+$S4_H$ was also a more potent prIFN activator which would fit the hypothesis that this virus had a decreased capping ability and therefore was sensed more efficiently by cellular pattern recognition receptors. Surprisingly, we did not detect decreased replication of $rgBTV8_H$ and on contrary to what we observed in OvEC, this virus produced at least 10 times higher titres than $rgBTV8_L$ in A549 cells. An important aspect that has to be considered when using human carcinoma cell lines to study BTV fitness and IFN related responses is whether this cell line can be used as an adequate model of host-pathogen interactions of the natural host. BTV has been shown to be a potent IFN inducer, especially in human transformed cell lines (Jameson and Grossberg, 1981). However, the levels of produced IFN varied depending on the type of cell, as well as the animal species (Russell et al., 1996; Vitour et al., 2014). This observation could be related to the intrinsic ability of specific cell lines to express IFN or to the virus being capable of interfering with IFN induction in particular lineages or species (Randall and Goodbourn, 2008; Spiropoulou et al., 2007). The role of the IFN system in cross-species barrier to viral infections is well established (Parrish et al., 2008; Randall and Goodbourn, 2008). A particularly suitable example here is the susceptibility of IFNAR knockout mice to BTV infection, while wild type mice are resistant. Similarly, humans are not susceptible to BTV and therefore specific restriction mechanisms must exist in human cell lines that are not found in sheep cell lines. Our data strongly suggested that A459 cells might not be the most suitable model to study BTV-host interactions. Hence, we did not find this cell line relevant for further exploration of the determinants of BTV pathogenesis.

In conclusion, our data shows that virulence of BTV-8 is a complex multifactorial phenomenon that is not attributed to a single protein only. Although we cannot exclude the possibility that silent mutations present in the original $BTV8_H$, but not in the rescued $rgBTV8_H$, contributed to the inability of $BTV8_H$ to replicate in the natural host, we found that amino acid mutations were sufficient to attenuate this virus. Interestingly, replacing only one or two segments of $rgBTV8_L$ with the corresponding segments from $rgBTV8_H$, were sufficient to attenuate the virus *in vivo*. However, at least five segments of the $BTV8_L$ strain had to be present for full virulence to be achieved. Given the high diversity of BTV, it is likely that different determinants of pathogenicity will be found in other serotypes. However, our data show that it is possible to design BTV vaccine strains rationally to minimise the probability of reversion to virulence in the field. Additional

investigations in the determinants of virulence of BTV will shed more light into the mechanisms of BTV replication and dissemination within the host in order to understand further why some strains (such as $BTV8_H$) do not induce viraemia in sheep or display delayed replication patterns. With the rapidly changing dynamics between climate, vector ecology and BTV, the understanding of virus virulence factors will be exceedingly important in order to understand and predict the risk posed by newly emerging BTV strains.

Chapter 5

Influence of genetic diversity of Bluetongue virus 8 on virulence

5.1 Introduction

Bluetongue is remarkably variable in its clinical outcomes (Anderson et al., 1985; Brenner et al., 2011; Maclachlan et al., 2009; Spreull, 1905). This variability has been attributed to host related factors such as species, breed, age and immune status and equally importantly, to virus facts related to differences between viral serotypes and strains (Caporale M., 2014; Maclachlan, 1994; Maclachlan et al., 2009; Oura et al., 2009; Parsonson, 1990; Waldvogel et al., 1987). Early studies looked at the pathogenicity of two strains of BTV-11, UC-2 and UC-8, which showed striking differences in their virulence in newborn BALB/c mice (Waldvogel et al., 1986). Both strains had the same *in vitro* passage history. However, they were isolated from a different animal species (UC-2 from calf, UC-8 from deer). Increased pathogenicity of UC-8 was later attributed to its ability to infect and spread in cells of neural origin and mapped to genetic differences in the VP5 protein (Waldvogel et al., 1987) (Carr et al., 1994). Experimental studies comparing the pathogenicity of different BTV strains in the natural host demonstrated that South African strains of BTV-1 and 3 caused significantly more severe disease than the Australian strains (Hooper et al., 1996). Another notable example is a strain of BTV-8 that emerged in Northern Europe in 2006 and spread through the continent causing high mortality in naïve sheep flocks and severe clinical disease in cattle (Elbers et al., 2008a; Elbers et al., 2008c; Perrin et al., 2010). However, no clinical signs in ruminantswere seen when BTV-8 appeared in North Italy in 2008.

Comparison of the virulence of European strains of BTV-8 and BTV-1 showed that the latter was significantly more pathogenic in the natural host (Sanchez-Cordon et al., 2013). However, passage history of both viruses in this study was omitted. Virus passage history has been reported to influence the clinical outcome of BTV infection in ruminants (Bonneau et al., 2002; Caporale et al., 2014; DeMaula et al., 2002b; Ghalib et al., 1985).

Our group used standardised experimental conditions to assess the pathogenicity of BTV-8 strains isolated in 2006 and in 2007 in the Netherlands (BTV-$8_{NET2006}$, BTV-$8_{NET2007}$) and in 2008 in Italy (BTV-8_{IT2008}) in the BTV natural host, the sheep (Caporale et al., 2014). All viruses used in the study had similar passage history in cell culture. Marked reduction in virulence was observed in animals infected with BTV-8_{IT2008} compared with the Dutch strains of BTV-8 (BTV-$8_{NET2006}$ and BTV-$8_{NET2007}$). BTV-8_{IT2008}-infected sheep showed only

mild transitory fever and no obvious clinical signs while both BTV-$8_{NET2006}$, BTV-$8_{NET2007}$ induced typical symptoms of bluetongue. The cumulative clinical scores recorded in animals infected with BTV-$8_{NET2006}$, BTV-$8_{NET2007}$ and BTV-8_{IT2008} were 58, 49 and 15, respectively (Caporale et al., 2014).

Genetic drift and especially reassortment occurring during epidemics plays a significant role in the diversification of BTV strains and their pathogenic potential (Nomikou et al., 2015). Experimentally, passaging of BTV in tissue culture was shown to have an impact on virulence *in vivo* (Caporale et al., 2011; Coetzee et al., 2012b; Moulin et al., 2012). In particular, strains isolated from severe clinical cases and subsequently adapted to mammalian tissue culture have been reported to have a reduced virulence in experimentally infected animals (Caporale et al., 2014). Some studies used therefore blood from vireamic animals as inoculum in the experimental setting in order to reproduce those severe clinical manifestations of bluetongue that are often seen in the field (MacLachlan et al., 2008; Moulin et al., 2012). In order to evaluate the effect of *in vitro* isolation of BTV-8 on its virulence *in vivo*, Caporale and colleagues inoculated two groups of Sardinian sheep with either blood from a BTV-infected animal (BTV-$8_{NET2007(blood)}$) or with the same virus but after isolation in KC cells and passaged twice in BHK-21 cells (BTV-$8_{NET2007(1KC-2BHK)}$). Sheep infected with BTV-$8_{NET2007(blood)}$ displayed more severe clinical symptoms, had greater fever and significantly higher viraemia compared with BTV-$8_{NET2007(1KC-2BHK)}$ (Caporale et al., 2014).

In this chapter, we aimed first to analyse the genetic changes that led to variation in virulence of BTV-$8_{NET2006}$, BTV-$8_{NET2007}$ and BTV-8_{IT2008}. We therefore fully sequenced the genomes of these viruses and compared their consensus sequences. In addition, we aimed to investigate the the genetic changes that might have occurred after isolation of BTV-$8_{NET2007(blood)}$ in cell culture.

5.2 Results

5.2.1 Influence of BTV-8 strain on clinical outcome of infection.

Experimental infection of sheep with three distinct strains of BTV-8 isolated in subsequent years (BTV-$8_{NET2006}$, BTV-$8_{NET2007}$ and BTV-8_{IT2008}) showed progressive gradation in their virulence (Caporale et al., 2014). BTV-8_{IT2008} was significantly less pathogenic than the strains from the Netherlands and induced only mild clinical signs of infection. To link the phenotypic differences described above to genetic mutations that might have emerged during the BTV-8 circulation between 2006 and 2008, we sequenced the genomes of BTV-$8_{NET2007}$ and BTV-8_{IT2008} and compared their sequences to BTV-$8_{NET2006}$. Compared to BTV-$8_{NET2006}$, BTV-$8_{NET2007}$ and BTV-8_{IT2008} had 26 and 24 nucleotide mismatches, respectively. Five mismatches were present in both strains (Figure 29). Amino acid mismatches were only found in four segments of BTV-$8_{NET2007}$ (S1, S4, S8 and S9). Interestingly, a mutation in nucleotide 413 of S9 lead to Ala to Val change in VP6 and at the same time introduced an early stop codon in the NS4 protein, resulting in a protein of 77 amino acid residues instead than 79. The presence of two stop codons in close proximity in the NS4 ORF suggests that this region is adapted to allow for variability in the VP6 ORF without affecting the NS4 structure. S10 was conserved between BTV-$8_{NET2006}$ and BTV-$8_{NET2007}$. In contrast, mismatches compared to BTV-$8_{NET2006}$ were found in all 10 segments of BTV-8_{IT2008} while non-synonymous mutations were concentrated in S1, S2, S4, S5, S8 and S9 (encoding VP1, VP2, VP4, NS1, NS2 and VP6, respectively). We have not been able to carry out experiments to identify which of these mutations led to decreased pathogenicity of the Italian strain compared to the strains isolated in the Netherlands. However, as amino acid changes in S8 position 59 and S9 position 5 occurred in both BTV-$8_{NET2007}$ and BTV-8_{IT2008}, these were unlikely responsible for the difference in their phenotypes *in vivo*.

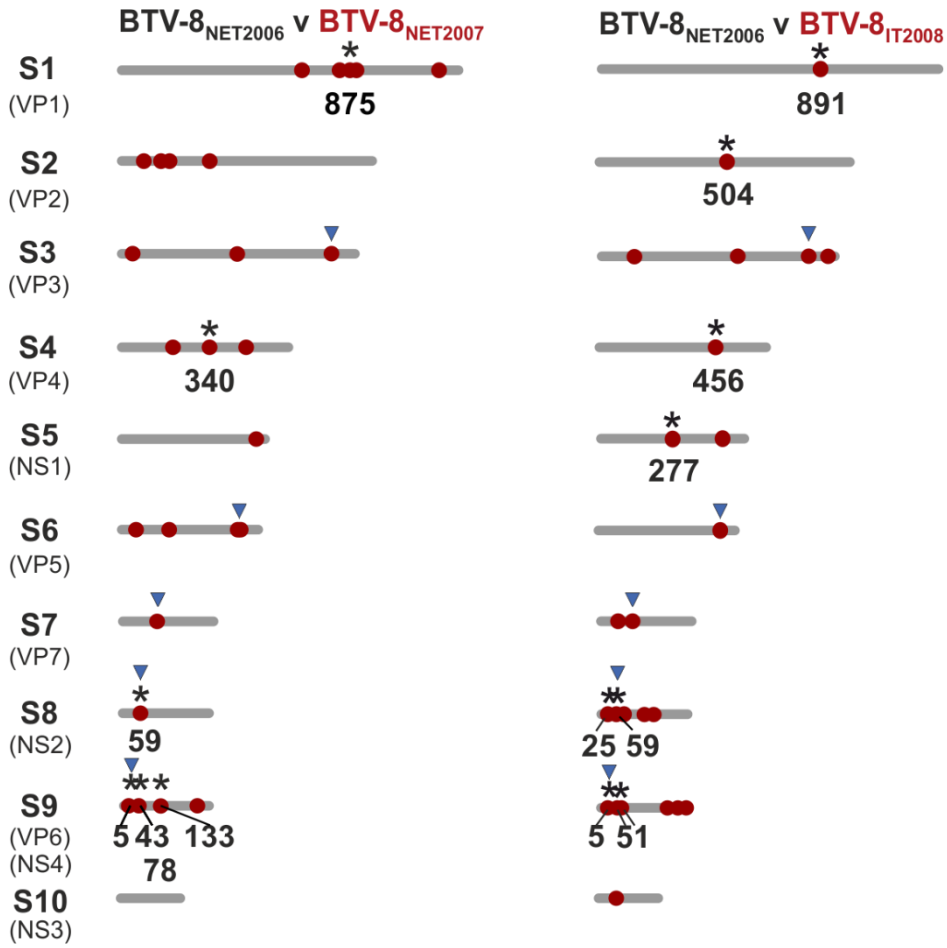

Figure 29. Genomic differences between BTV-$8_{NET2006}$, BTV-$8_{NET2007}$ and BTV-8_{IT2008}. Schematic representation of the 10 genomic segments of BTV. Mutations in BTV-$8_{NET2007}$ or BTV-8_{IT2008} compared to BTV-$8_{NET2006}$ are indicated with red dots. Non-synonymous mutations are marked with asterisks and the numbers relative to the mutated amino acid residue in the corresponding viral proteins are shown. Blue triangles mark mutations that are present in both BTV-$8_{NET2007}$ and BTV-8_{IT2008}. Drawings (length of the schematic genome segments and relative position of mutations) are indicative only.

5.2.2 Influence of passage history on BTV population diversity.

Previous experiments performed by our group showed that sheep inoculated with infectious blood carrying BTV-$8_{NET2007}$ (termed BTV-$8_{NET2007(blood)}$) displayed more severe clinical signs than animals infected with the same virus passaged once in KC cells and twice in BHK-21 cells (BTV-$8_{NET2007(1KC-2BHK)}$) (Caporale et al., 2014). In order to find if these phenotypic differences were associated with genetic changes we analysed the genomes of BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK)}$ by deep sequencing. RNA of both viruses was isolated from (i) the same inoculum (i.e. blood) that was used in *in vivo* experiments described above, (ii) after isolation in KC cells (BTV-$8_{NET2007(1KC)}$), (iii) after one (BTV-$8_{NET2007(1KC-1BHK)}$) and two further passages in BHK cells BTV-$8_{NET2007(1KC-2BHK)}$. Furthermore, we independently repeated the isolation of BTV-$8_{NET2007}$ from infected tissue in KC cells followed by two passages in BHK-21 cells and sequenced the resulting viruses. Hence, deep sequencing of seven samples was carried out which included BTV-$8_{NET2007(blood)}$, and two independent isolates of BTV-$8_{NET2007(1KC)}$, BTV-$8_{NET2007(1KC-1BHK)}$ and BTV-$8_{NET2007(1KC-2BHK)}$.

We did not find any amino acid mismatches between the consensus sequences of BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK)}$ (Figure 30A). Two synonymous mutations, in segments 1 (nt 2756) and segment 4 (nt 1431) however, were found in BTV-$8_{NET2007(1KC-2BHK)}$ and these were selected after the initial passage in KC cells and in both experiments. To understand how the same mutations could have emerged in two independent replicates we examined the proportion of reads in BTV-$8_{NET2007(blood)}$ that contained either the original or mismatched nucleotide. We found that more than 10% of reads in BTV-$8_{NET2007(blood)}$ sample contained an adenine in position 2756 of S1 (14.9% of reads) and a thymine residue in position 1431 of S4 (10.4% of reads) (Figure 30B). These minority variants were then selected after the first passage in tissue culture.

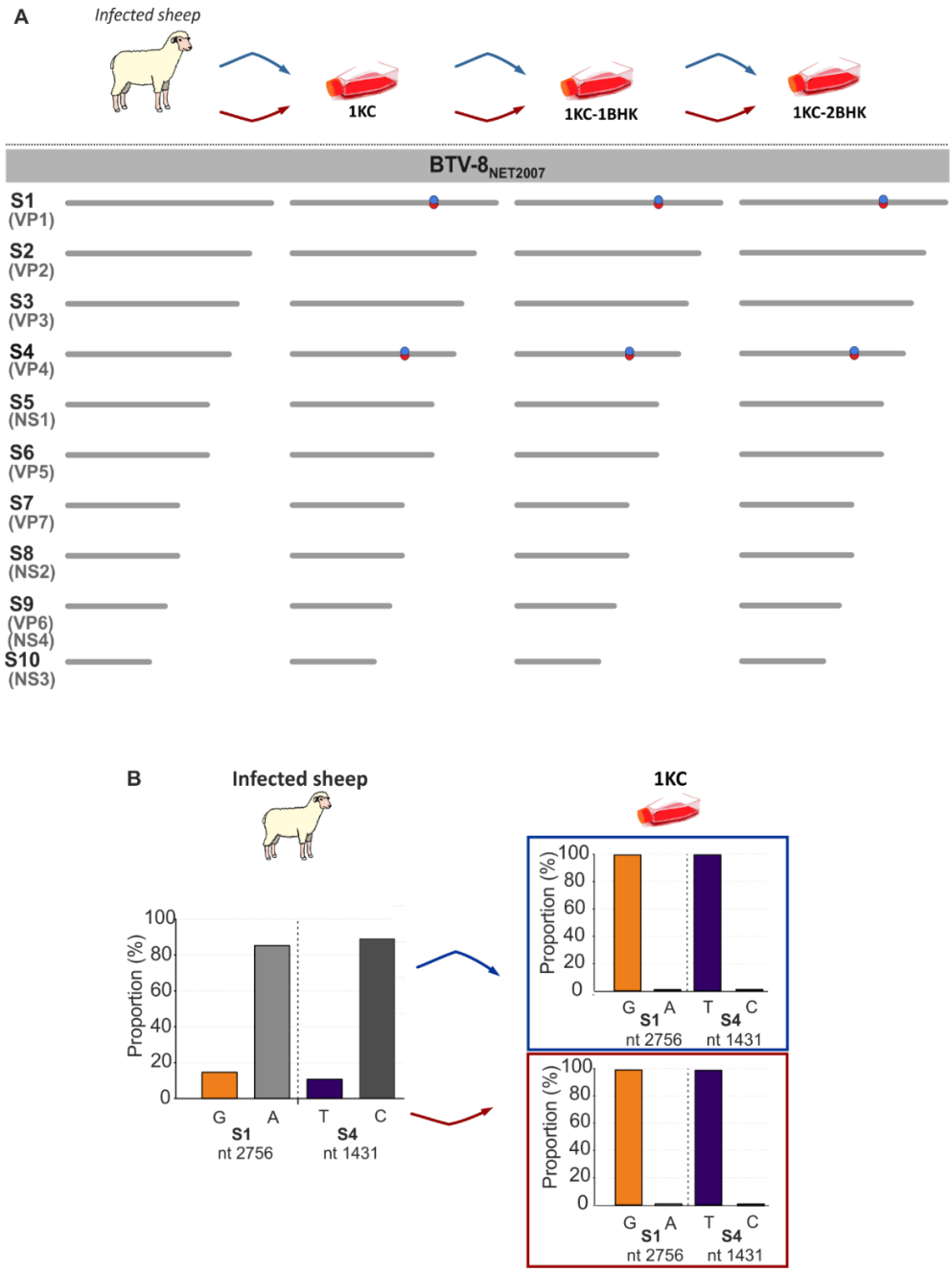

Figure 30. Isolation of BTV-8$_{NET2007(blood)}$ *in vitro*. The effects of adaptation *in vitro* of BTV-8$_{NET2007(blood)}$ was assessed by comparing the genomic sequences of BTV-8$_{NET2007(blood)}$ with the sequences of viruses isolated *in vitro* after passaging in *Culicoides* KC cells (1 passage) and two further passages in BHK-21 cells (A). Two independent isolations (represented with blue or red arrows) were carried out and genome sequences were obtained after each passage in cell culture. The cartoon shows the schematic representation of individual genomic segments of BTV. Mutations found in the consensus sequences of the cell culture passaged viruses are shown as red or blue dots indicating the two independent experiments. Two synonymous mutations were selected in Seg-1 and Seg-4 immediately after passage in KC cells in both independent experiments and were conserved after further passaging in BHK-21 cells. (B) Analysis of nucleotide variants at the mutation site revealed mixed population in BTV-8$_{NET2007(blood)}$ and the minority variants were selected in both independent experiments.

RNA viruses have very high mutation rates and as such they exist as a 'cloud' of variants (or quasispecies), each possessing random nucleotide mismatches and often different phenotypes (Lauring and Andino, 2010; Lauring et al., 2013; Vignuzzi et al., 2006). Since we did not detect any amino acid mismatches between consensus sequences of BTV-$8_{NET2007(blood)}$ and tissue culture adapted viruses and yet we saw clear differences in the severity of disease in sheep, we hypothesised that *in vitro* passage affected viral population diversity. We analysed the single nucleotide variation present in BTV-$8_{NET2007}$ before and after passage in KC and BHK-21 cells. We re-mapped reads to the established consensus sequences and analysed variability at each nucleotide position for all ten genomic segments. Figure 31 shows the degree of variability that occurred at each site of the genome before and after passage in cell culture. A 'variant' was called (and plotted in the graph) if it was present in at least 0.1% of mapped reads at a specified site and in at least 0.1% of the virus population. In general, the number of variants dramatically decreased after passage in mammalian cells. Interestingly, for 9 of the 10 segments in the first set of experiments, and for 8 of the 10 segments in the second set of experiments, the number of variable nucleotides was higher in the virus passaged once in KC cells than in the virus from blood before passage in cell culture. Additionally, in all samples passaged *in vitro* S1 (VP1) had the highest percentage of detected variants (Table 7).

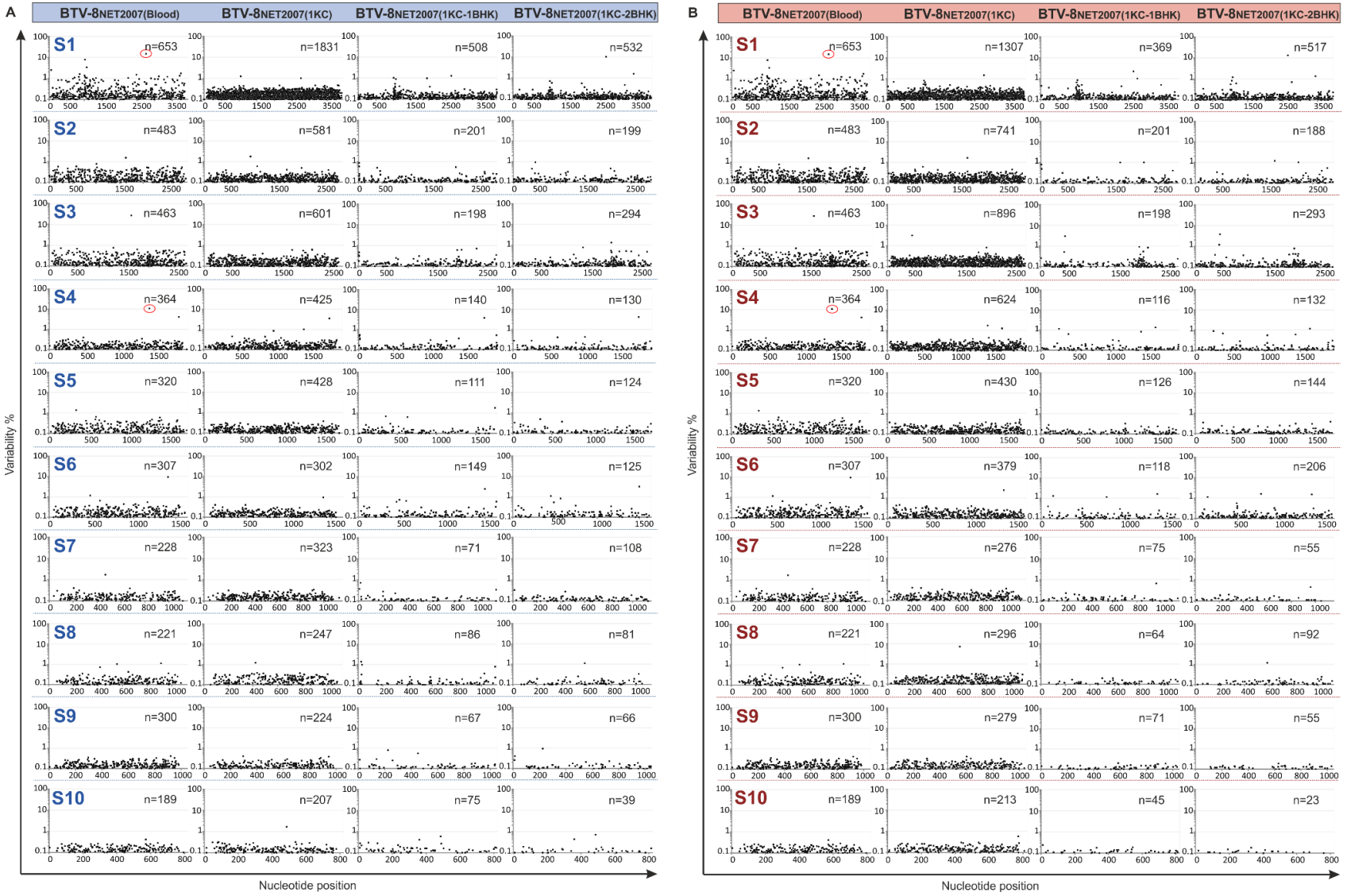
A
BTV-8NET2007(Blood)
BTV-8NET2007(1KC)
BTV-8NET2007(1KC-1BHK)
BTV-8NET2007(1KC-2BHK)
S1 n=653 n=1831 n=508 n=532
S2 n=483 n=581 n=201 n=199
S3 n=463 n=601 n=198 n=294
S4 n=364 n=425 n=140 n=130
S5 n=320 n=428 n=111 n=124
S6 n=307 n=302 n=149 n=125
S7 n=228 n=323 n=71 n=108
S8 n=221 n=247 n=86 n=81
S9 n=300 n=224 n=67 n=66
S10 n=189 n=207 n=75 n=39
Variability %
Nucleotide position
B
BTV-8NET2007(Blood)
BTV-8NET2007(1KC)
BTV-8NET2007(1KC-1BHK)
BTV-8NET2007(1KC-2BHK)
S1 n=653 n=1307 n=369 n=517
S2 n=483 n=741 n=201 n=188
S3 n=463 n=896 n=198 n=293
S4 n=364 n=624 n=116 n=132
S5 n=320 n=430 n=126 n=144
S6 n=307 n=379 n=118 n=206
S7 n=228 n=276 n=75 n=55
S8 n=221 n=296 n=64 n=92
S9 n=300 n=279 n=71 n=55
S10 n=189 n=213 n=45 n=23
Variability %
Nucleotide position

Figure 31. Viral population diversity of BTV-8NET$_{2007(blood)}$ before and after isolation *in vitro*. Changes in nucleotide diversity of BTV-8$_{NET2007(blood)}$ amplified directly from the spleen of an infected sheep were compared with sequences of the same virus after isolation in KC and BHK-21 cells. Differences were assessed by deep sequencing as described in Materials and Methods. Total reads of individual genome segments were mapped to consensus sequences and single nucleotide polymorphisms (SNPs) were assigned above the arbitrary 0.1% frequency threshold. On the graph each dot represents the percentage of nucleotides difference (y-axis) from the consensus sequence of each nucleotide composing the individual genomic segments of the virus (x-axis). The total number of variable nucleotides (> 0.1%) for each sample is shown in the right corner of each plot. Dots circled in red in Seg-1 and Seg-4 of BTV-8$_{NET2007(blood)}$ are those nucleotides that have been selected in the majority of the viral populations after passage in vitro. Virus isolation was performed twice independently and the variant detection analysis was performed for the first (A) and second (B) replicates separately.

Table 7. Percentage of variable nucleotides (SNP>0.1%) in genome of BTV-$8_{NET2007(blood)}$ before and after passage *in vitro.*

Segment	BTV-8 NET2007(blood)	BTV-8 NET2007(1KC)	*Replicate 1* BTV-8 NET2007 (1KC-1BHK)	BTV-8 NET2007 (1KC-2BHK)	*Replicate 2* BTV-8 NET2007(1KC)	BTV-8 NET2007 (1KC-1BHK)	BTV-8 NET2007 (1KC-2BHK)
1	16.51	45.85	12.85	13.45	33.06	9.33	13.08
2	16.51	19.86	6.87	6.80	25.32	5.26	6.43
3	16.71	21.70	7.15	10.61	32.35	7.15	10.58
4	18.37	21.45	7.07	6.56	31.50	5.86	6.66
5	18.09	24.19	6.27	7.01	24.31	7.12	8.14
6	18.74	18.44	9.10	7.63	23.14	7.20	12.58
7	19.72	27.94	6.14	9.34	23.88	6.49	4.76
8	19.66	21.98	7.65	7.21	26.33	5.69	8.19
9	28.68	21.41	6.41	6.31	26.67	6.79	5.26
10	22.63	25.18	9.12	4.74	25.91	5.47	2.80
Total	19.56	24.80	7.86	7.97	27.25	6.64	7.85

Low frequency variants (0.1 – 0.29%) were most abundant in BTV-$8_{NET2007(1KC)}$, while the number of variants with a frequency of >0.4% was several fold higher in BTV-$8_{NET2007(blood)}$ (Figure 32). We detected only a few nucleotides with >10% variability and these included the above mentioned variants in S1 and S4 as well as two others in BTV-$8_{NET2007(blood)}$ (S3 and S6) that were not selected for *in vitro*. The majority of viral species had a thymine at position 1644 of S3 and position 1407 of S6, while minority variants had a cysteine instead. This variation did not lead to amino acid mismatches.

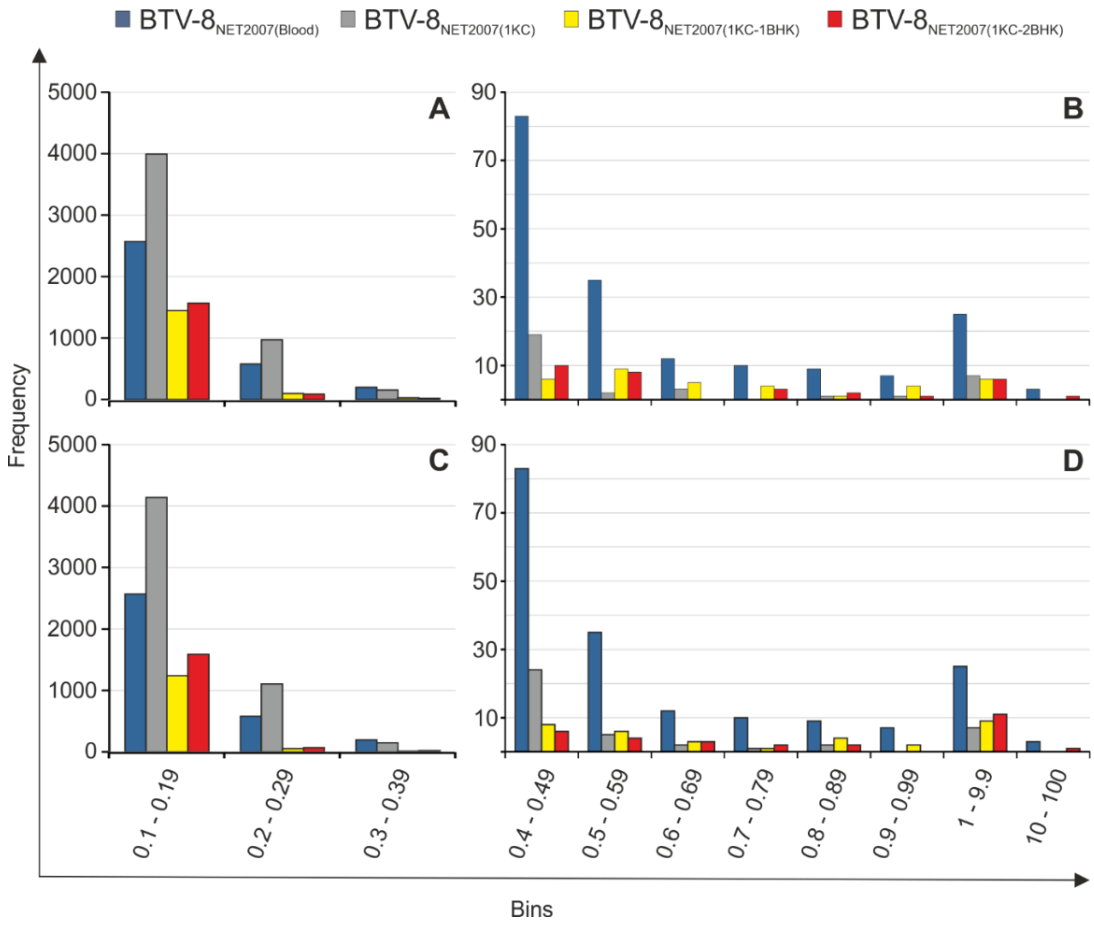

Figure 32. Frequency distribution of variable nucleotide in BTV-8$_{NET2007(blood)}$, BTV-8$_{NET2007(1KC)}$, BTV-8$_{NET2007(1KC-1BHK)}$ and BTV-8$_{NET2007(1KC-2BHK)}$. Histograms showing for each virus the number of nucleotides with percentage variation falling within defined bins. Panels A-B and C-D represent data of two independent experiments. Note that panels B and D have a different scale in the y-axis compared than panels A and C as the frequency of variants present in more than 0.4% of the total population was significantly lower compared to variants presented in panels A and C.

5.3 Discussion

BTV-8 has emerged in central Europe in 2006 and subsequently spread to neighbouring areas, reaching Northern Italy in 2008. Two strains of BTV-8 used in this study were isolated from viraemic animals in 2006 and 2007 in the Netherlands. It has been reported that the 2007 epidemics in the Netherlands involved a larger number of animal herds than the 2006 outbreak, and consequently led to higher morbidity and mortality. The disease severity reported in the field was similar for both strains, although more cases of lameness were observed in sheep in 2007 (Elbers et al., 2009). Experimental studies done by our group using BTV-$8_{NET2006}$ and BTV-$8_{NET2007}$ with similar *in vitro* passage history confirmed that both viruses were highly virulent in sheep, although BTV-$8_{NET2006}$ achieved slightly higher clinical scores in all tested categories (respiratory, general, fever and total clinical score) (Caporale et al., 2014). On the contrary, BTV-8_{IT} was not associated in the field with overt clinical symptoms and was detected only due to several animals seroconverting to this serotype. In experimental conditions, BTV-8_{IT2008} was shown to be less virulent than both strains isolated in the Netherlands (Caporale et al., 2014). Deep sequencing of all three strains showed BTV-8_{IT2008} had over 20 mismatches compared with BTV-$8_{NET2006}$ and these included nonsynonymous changes in six segments. Four of these segments (S1, S2, S4, and S9), had already been identified by our group as major determinants of BTV virulence (Chapter 4), while S8 (as well as S1 and 2) has been shown to be consistently mutated in tissue culture attenuated strains of BTV-2, BTV-4 and BTV-9 (Caporale et al., 2011). We found five common mismatches when we compared both BTV-$8_{NET2007}$ and BTV-8_{IT2008} with BTV-$8_{NET2006}$. BTV-$8_{NET2007}$ retained similar *in vivo* phenotype to BTV-$8_{NET2006}$ (despite 7 coding and 19 silent mutations) while BTV-8_{IT2008} became much less pathogenic. It is unlikely that the amino acid substitutions present both in BTV-$8_{NET2007}$ and BTV-8_{IT2008} played a role in their pathogenicity, although we cannot exclude this possibility. In the previous chapter, we demonstrated that combinations of mutations in several segments are involved in BTV-8 virulence *in vivo*. Hence, a similar approach will need to be considered in order to determine which genomic segments, or individual mutations, are responsible for the different virulence possessed by BTV-8_{IT2008} and BTV-$8_{NET2006}$.

Passage of a wild type virus *in vitro* often leads to genetic changes that facilitate entry and replication in specific cell lines without constraints from the host antiviral immune responses. Hence, tissue culture adaptation can alter viral phenotype and consequently

change its virulence *in vivo*. Experimental infection of sheep with BTV-$8_{NET2007(blood)}$ and BTV-$8_{NET2007(1KC-2BHK)}$ showed that the latter produced less severe disease manifestations. However, comparison of consensus sequences of both viruses showed only two nucleotide mismatches that did not lead to amino acid changes. These variants were already present in 10% of the viral pool in BTV-$8_{NET2007(blood)}$ and were independently selected in two experiments. Very little is known about the determinants of BTV pathogenicity and the involvement of synonymous mutations in BTV virulence. It is possible that these mutations could influence BTV RNA structures and thus affect its fitness (Feenstra et al., 2014). In addition, since our sequencing approach involved amplification of individual segments using specific primers overlapping the 3' and 5' end of each genomic segment, it is possible that we could have missed some changes in the untranslated regions of the virus genome that might have affected BTV-$8_{NET2007(1KC-2BHK)}$ virulence (Lymperopoulos et al., 2003; Sung and Roy, 2014). Nonetheless, our experiment showed that isolation and initial passages of BTV-8 *in vitro*, have little overall effect on consensus sequence of BTV-8 and it appears that the selection of specific variants happens immediately after the first passage in KC cells.

Our data demonstrate that high frequency variants (>10%) were the most abundant in BTV$8_{NET2007(blood)}$ but polymorphisms in different nucleotides also started to emerge in BTV-$8_{NET2007(1KC)}$ as well. Surprisingly, two independent experiments showed that variation across the entire genome was the highest in both BTV-$8_{NET2007(1KC)}$ samples (24.8% and 27.25%), followed by BTV-$8_{NET2007(blood)}$ (19.56%). These results suggest that BTV-8 replication in insect cells leads to amplification of a number of emerging variants and as a result, to the generation of a wide spectrum of viral progeny genetically divergent from the consensus sequence. These data are in accord with Jerzak *et al.* who compared genetic diversity of West Nile virus passaged multiple times in either birds or mosquitos and concluded that while virus population passaged in chickens was more homogenous, higher number of quasispecies was observed in insect passaged virus (Jerzak et al., 2007; Jerzak et al., 2008). Moreover, at least double the amount of variation was observed in naturally infected mosquitoes than in the avian host. In light of these data, the authors concluded that insect vectors served as sources of genetic variation of the virus while the vertebrate host was a "selective sieve" that reduced viral population diversity (Jerzak et al., 2005). Only few studies looked at changes of BTV genetic variability in insect and mammalian hosts. Bonneau *et al.* showed that transmission of BTV-10 between ruminants and *Culicoides* midges led to an

increased number of variants in S2 and S10 (coding for VP2 and NS3, respectively) but the consensus sequence remained unchanged (Bonneau et al., 2001).

The factors accounting for an increased number of variants in the BTV population found in insect cells are not clear at present. One of the more recognised causes behind increased quasispecies generation is the decreased polymerase fidelity (Lauring and Andino, 2010). Early studies looking into the efficacy of BTV RdRp (VP1) showed that the optimal transcription of the BTV genome occurred at 28^{o}C but this threshold could be shifted upwards by altering the sugar concentration in the medium (Van Dijk and Huismans, 1980, 1982). The temperature in insects is lower than 30^{o}C and KC cells were maintained in our studies at 28^{o}C. On the other hand, sheep body temperature exceeds 38^{o}C while mammalian cell cultures used in our study are maintained at 37^{o}C. Hence, it is possible that the activity of VP1 is affected by the temperature in the host organism and consequently its fidelity might be dramatically different between the insect and the mammalian hosts.

In addition, factors affecting the interaction of the virus with the insect cell could also explain the high number of observed variants in KC cells, as opposed to host or mammalian cells. Several proteins including NS4 and NS3 have been shown to interact with the host cell antiviral response (Chauveau et al., 2013; Ratinier et al., 2011). Hence, changes on the amino acid level could influence the ability of the virus to counteract defences of the mammalian host cell. However, insect cells do not have an IFN system and instead rely on interference RNA (RNAi) to combat invading pathogens (Kingsolver et al., 2013). An interesting hypothesis could therefore be that the generation of a larger spectrum of variants would be a means to escape highly specific RNAi species. Other, virus unrelated factors, e.g. cellular RNA editing enzymes could also play role in increased/decreased variant numbers in different types of samples (Liu et al., 2014; Rima et al., 2014; Zahn et al., 2007).

Alternatively, it is possible that the number of virus replication cycles was higher in KC than in BHK-21 cells, raising the number of events when polymerase errors occurred, which consequently led to the generation of larger amount of variants. Moreover, passaging virus at high MOI often results in larger population size (Froissart et al., 2004; Lauring et al., 2013; Montville et al., 2005). In our study, we passaged the virus blindly and therefore did not control the titre of virus in the inoculum nor in the cell lysate used for the isolation of dsRNA. Future work looking at expansion of BTV population using a defined virus concentration in a

homogenous inoculum (e.g. strain rescued with reverse genetics) and during virus passage would therefore provide more insight into mechanisms of BTV quasispecies generation and limitations of using specific types of inocula for *in vivo* experiments.

Our data clearly indicate that a dramatic bottleneck in BTV transmission happened in mammalian cells. Additionally, very few CPE foci were observed initially after BHK-21 cells were infected with infectious supernatant from KC cells (data not shown). These foci likely represented the rare variants that were able to efficiently infect and replicate in BHK-21 cells. It has been shown previously that high-fidelity mutants of Poliovirus generate low diversity progeny population and loses neurotropic properties *in vivo* (Vignuzzi et al., 2006). Interestingly, the generation of higher quasispecies diversity through chemical mutagenesis restores virulence in the mouse model despite having the same consensus sequence as the attenuated virus. Several other studies confirmed that genetic heterogeneity of the quasispecies should be considered as an important determinant of pathogenicity in non-clonal virus populations (Ebel et al., 2011; Farci et al., 2002; Sauder et al., 2006) (Clarke et al., 1993; Coffey and Vignuzzi, 2011). This could explain why we detected a decrease in virulence in sheep inoculated with BTV-$8_{NET2007(1KC\text{-}2BHK)}$ compared with animal blood despite having only two nucleotide mismatches in the consensus sequence. In BTV-$8_{NET2007(1KC\text{-}2BHK)}$ less than 8% of genome showed variability of 0.1% or more, while in BTV-$8_{NET2007(blood)}$ this number was ~20%. A more diverse BTV virus cloud could therefore be a factor in increased pathogenicity of infectious blood compared with the sample passaged in tissue culture.

In summary, the clinical outcome of BTV infection is determined by both host and virus factors. Within the virus factors, the severity of the disease depends on the infecting strain (but not necessarily from the serotype). However, the virulence is not only affected by genetic changes at the consensus level but also by the overall genetic diversity of the viral population (quasispecies). Although the majority of randomly generated variants will carry deleterious or fatal mutations, the remaining 'viral cloud' might contain specific variants with the ability to cross specific infection barriers of the host or the vector. Our study reveals many questions about the importance of BTV quasispecies in nature and the role of the vector/host cycle in the maintenance of variant heterogeneity that should be addressed in order to gain a further understanding of basic BTV biology.

Chapters 6
FINAL CONCLUSIONS

One of the basic biological characteristics of a virus is its ability to cause disease (or lack of thereof), which is referred to as pathogenicity (Casadevall and Pirofski, 2001; Dortmans et al., 2011). The degree of pathogenicity can be "quantified" further in terms of virulence, which is usually associated with the clinical outcome or severity of infection. Therefore, although pathogenicity is a property of a virus, it can only be measured *in vivo*, in the context of an appropriate host and specific virus-host interactions. This poses a particular problem for viruses such as BTV, which exist as multiple serotypes/strains and can infect several animal species/breeds. Indeed, although BTV has been extensively studied for decades, there is very little concrete evidence of specific viral determinants of its virulence. Moreover, the established mouse models do not fully mirror the virus/host interplay in ruminants, which further hinders research on bluetongue pathogenesis. Nonetheless, an identification of the molecular determinants of viral pathogenicity is essential in order to: 1) gain basic understanding of BTV biology (e.g. host/vector range, transplacental spread, potential for rapid transmission, strain virulence); 2) devise control measures that fit the risks posed by specific strains/serotypes; 3) generate safe and efficacious vaccines based on truly attenuated strains that cannot be transmitted by vectors and cannot reassort in the field or revert to virulence.

In our study, we used several models of infection to assess virulence and the fitness of parental $BTV8_H$ and $BTV8_L$ and reassortant viruses. The viruses had different phenotypes in the natural host. While $BTV8_L$ was highly pathogenic in sheep, $BTV8_H$ was fully attenuated and did not cause either detectable viraemia or clinical signs in infected animals. In order to understand roles of specific genomic segments in determining BTV-8 pathogenicity *in vivo* we therefore generated reassortants between both parental viruses and tested them in $IFNAR^{-/-}$ mice. It is necessary however to recognize the limitations of $IFNAR^{-/-}$ mice as a model of BTV infection. Due to the lack of a functional IFN system, $IFNAR^{-/-}$ mice are particularly susceptible to infection with wild type BTV-8 and infection even at very low doses will invariably lead to animal death. In contrast, in the field, mortality due to BTV rarely exceeds 30%, partially due to specific immune responses mounted by the natural host (Schultz and Grieder, 1987). Moreover, certain strains of BTV, e.g. $BTV8_{IT2008,}$ are only mildly pathogenic in ruminants but cause 100% mortality in mice, which shows that certain factors might be overlooked when studied exclusively in the mouse model. Similarly, a BTV-8 NS4 deletion mutant replicates with the same efficiency as wild type BTV *in vitro*, and is equally virulent

in IFNAR$^{-/-}$ and newborn mice yet in sheep it is attenuated (Ratinier et al., 2011) and (Ratinier *et al.*, unpublished). Our study showed that BTV8$_H$ was non-pathogenic in IFNAR$^{-/-}$ mice as well as in the natural host. However, the effect of specific segment mutations on the clinical outcome of infection could not be tested in sheep. It is very likely that the combination of mutations that conferred fully attenuated phenotype in the mouse model (mutations in S2 and S1, S6 or S10) would likewise render the resulting reassortants non-pathogenic in the sheep as well. It is however also possible that some other combinations that were not identified in mouse studies would be sufficient for BTV attenuation in the natural host. Additional experiments in the ruminant host using selected reassortants would therefore further complement our work and provide further details on the roles of specific determinants in BTV pathogenesis.

Reverse genetics allows the generation of viruses with artificial genetic modifications that might not have arisen in the field, for example due to constraints imposed by the insect vector-mammalian host transmission boundaries. Similarly, tissue-culture attenuated strains might acquire features that would render them not viable in natural conditions. Even though such mutants might be good indicators of the importance of specific gene products in replication and host interactions, their use for the prediction of virulence potential has to be taken with caution. For example, based on our studies of BTV8$_H$ fitness *in vitro* and virulence in the mouse model we predicted that mutations in at least six proteins contributed to its attenuated phenotype, with VP2 being the main determinant. However, increased affinity of VP2$_H$ for GAGs is a result of tissue-culture passage and is unlikely to arise and contribute to strain pathogenicity in the field. While artificially generated characteristics might therefore tell us something about the biology of BTV, they might be less informative in determining factors contributing to the broad spectrum of *in vivo* phenotypes displayed by various strains/serotypes in the field. It would therefore be interesting to include in our analysis the mutations that we identified in BTV8$_{IT2000}$ as these occurred as an effect of natural genetic drift and resulted in decreased virulence in ruminants.

Viral pathogenicity is often multigenic and largely determined by specific gene constellations. For viruses with segmented genomes, genetic drift combined with genetic shift can lead to the generation of a variety of viruses with altered phenotypes. The polygenic nature of virulence has been particularly well studied in influenza A viruses, which similarly to BTV exists as multiple serotypes/strains that can differ significantly in their virulence

(Chen et al., 2008; Perez-Cidoncha et al., 2014). Similarly, multiple genes contribute to the pathogenicity of different members of the *Reoviridae* in animal models. Hoshino and colleagues showed that to achieve virulence in piglets it was necessary to replace four genes (VP3, VP4, VP7 and NSP4) of human Rotavirus strain DS-1 with analogous porcine genes (Hoshino et al., 1995). Single, double or triple segment reassortants were not capable of producing disease in this model (Hoshino et al., 1995). Rotavirus VP4 and NSP1 were also found to be the main factors associated with the murine intestine tropism, while a gene constellation including the VP3, NSP1, NSP3 and NSP2 determined the titre of virus produced in the intestine (Feng et al., 2013). A similar study looking into the determinants of pathogenicity of another member of the *Reoviridae*, Reovirus, demonstrated that L1, L2, M1, and S1 segments together contributed to the severity of disease in the mouse, while specific genes (or combinations) were responsible for particular tissue tropism (Haller et al., 1995). The data obtained in our work showed that, likewise, multiple genes govern BTV-8 virulence. Some of the proteins e.g. VP2 and NS3 determine BTV pathogenicity likely due to specific functions they play in virus/host interactions and therefore have the most dramatic effect viral virulence. Other proteins might have no effect on their own, but in combination with other factors can influence viral phenotype. It is likely that some of the segment combinations resulted in reassortants containing proteins that were not fully compatible with one another. For example, mismatches in segment 7 (VP7) alone did not have any effect on BTV8 pathogenicity or fitness, however the addition of $S7_L$ to a construct of $BTV8_H$+$S2/6/10_L$ resulted in increase of $IFNAR^{-/-}$ mortality from 20% to 60% at the 3000 PFU inoculation dose. Several studies showed that reassortment between particular BTV serotypes might not be fully flexible and preferential combinations might occur in a co-infected host or a vector (Nomikou et al., 2015; Ramig et al., 1989; Samal et al., 1987b). Nunes *et al.* attempted to rescue a full set of reassortants containing BTV-1 backbone and VP2 of 26 serologically different reference strains but did not succeed in rescuing eight of the proposed combinations (Nunes et al., 2014). Shaw *et al.* on the other hand showed that reassortment between all segments of BTV-1 and BTV-8 is flexible, however during mixed infection in vitro, certain combinations are recovered more often than others (Shaw et al., 2013). These studies clearly show that interactions between BTV proteins require specific molecular patterns (or sequences) and mismatches within interacting regions can result in suboptimal virion formation and release, and consequently affect virulence. This opens the possibility of using reverse genetics to generate viruses that are not able to reassort due to

strong structural affinity for specific protein sequences. Such a property would be important for MVL vaccines and as it would minimize the risk of reassortment with field strains.

Another study investigating the multigenic nature of Rotavirus pathogenicity, compared sequences of the EB strain passaged in either mice or in tissue culture (Tsugawa et al., 2014). Their results showed that mutations in at least three genes (VP4, NSP4 and NSP1) are associated with virulence in mice (Tsugawa et al., 2014). However, mutations in these proteins consistently appeared in viruses after *in vivo* passage and then disappeared during serial passage in cells. The authors suggested that the observed amino acid changes in the consensus could have been the result of particular quasispecies variants being selected in a specific passage system, rather than arising *de novo*. In Chapter 5, we showed that isolation of BTV-8 in insect cells followed by passage in mammalian cells decreases the number of low frequency variants in the sample pool, effectively reducing the size of "virus cloud" surrounding the consensus type. Furthermore, this change has an effect on virus virulence *in vivo*. A heterogeneous population has an advantage over a clonal one, as it contains a selection of viruses, that can freely reassort and use this "mix & match" system to adapt to diverse environments encountered in the vector, in the host, and also in diverse tissues within an animal. To date, there are no data available on the different bottlenecks imposed on the virus during its natural transmission cycle. Moreover, the mechanism of generation of variant diversity, other than random polymerase errors have not been established. Further studies looking into the role of the insect vector as a generator of a broad spectrum of BTV quasispecies would therefore be of great interest.

Our study shows that BTV-8 pathogenesis is governed by a combination of factors. These include major molecular determinants involved in direct interactions with the host and in the structure and functioning of the virion itself. Moreover, virulence is affected by the heterogeneity of the variants within a single strain population. It is necessary to point out that the specific factors might differ for other BTV serotypes/strains and therefore future studies examining conserved determinants would be of great interest. Such work would enable the generation of candidate MLV viruses that display high fitness in IFN-deficient systems but are unable to cause viraemia and induce clinical symptoms in the host. Additionally, synthetic high fidelity RdRp strains could prove effective in minimizing MLV genetic drift and reversion to virulence and therefore improve further BTV control measures.

Appendix

Table 8. Genetic differences between $BTV8_L$ and $BTV8_H$.

Segment	Protein	Nucleotide position	$BTV\text{-}8_L$ nucleotide	$BTV\text{-}8_H$ nucleotide	Amino acid position	$BTV\text{-}8_L$ amino acid	$BTV\text{-}8_H$ amino acid
1	VP1	3702	G	A	1231	Asp	Asn
2	VP2	63	G	A	16	Glu	Lys
		981	A	G	322	Asn	Asp
		996	A	G	327	Ile	Val
		1215	A	T	400	Arg	Trp
		1874	G	A			
		2060	A	G			
3	VP3	1091	A	G			
		1097	T	C			
		1994	T	C			
		2428	C	T	804	Thr	Met
4	VP4	1002	G	A	332	Asp	Asn
		1705	C	A	566	Thr	Ile
5	NS1	85	C	T			
		712	T	A	266	Asn	Lys
		1000	T	C			
		1401	C	T	456	Ala	Val
		1540	G	A			
6	VP5	12-13		+GC			
		252	A	G			
		1009	T	A	328	Phe	Ile
		1216	T	C			
7	VP7	843	C	T	276	His	Tyr
		999	G	A	328	Ala	Thr
8	NS2	92	C	A	25	Ala	Thr
		1078	C	T			
		1106	G	A			
9	VP6	955	A	G	314	Thr	Ala
		1022	C	T			
	NS4						
10	NS3	308	C	T	97	His	Tyr
		319	G	A			

Table 9. Genetic differences between BTV-$8_{NET2006}$ and BTV-$8_{NET2007}$.

Segment	Protein	Nucleotide position	BTV-$8_{NET2006}$ nucleotide	BTV-$8_{NET2007}$ nucleotide	Amino acid position	BTV-$8_{NET2006}$ amino acid	BTV-$8_{NET2007}$ amino acid
1	VP1	2051	T	C			
		2534	T	C			
		2634	G	A	875	Thr	Ala
		2756	G	A			
		3701	T	C			
2	VP2	242	G	A			
		443	A	G			
		470	T	C			
		1007	A	C			
3	VP3	113	T	C			
		1328	C	T			
		2471	T	C			
4	VP4	620	A	G			
		1026	T	A	340	Met	Leu
		1431	T	C			
5	NS1	1639	T	C			
6	VP5	144	G	A			
		546	G	A			
		1365	T	C			
		1383	G	A			
7	VP7	392	C	T			
8	NS2	194	A	G	59	Asp	Asn
9	VP6	30	A	G	5	Ile	Met
		142	A	G	43	Thr	Ala
		413	C	T	133	Ala	Val
	NS4	413	A	G	78	Gln	STOP
10	NS3	---					

Table 10. Genetic differences between BTV-$8_{NET2006}$ and BTV-8_{IT2008}.

Segment	Protein	Nucleotide position	BTV-$8_{NET2006}$ nucleotide	BTV-8_{IT2008} nucleotide	Amino acid position	BTV-$8_{NET2006}$ amino acid	BTV-8_{IT2008} amino acid
1	VP1	2683	C	T	891	Thr	Met
2	VP2	1527	C	T	504	His	Tyr
3	VP3	395	C	T			
		1667	C	T			
		2471	C	T			
		2645	A	G			
4	VP4	1375	T	C	456	Val	Ala
5	NS1	865	G	A	277	Met	Ile
		1426	G	A			
6	VP5	1383	A	G			
7	VP7	170	T	C			
		392	T	C			
8	NS2	93	C	T	25	Ala	Val
		194	G	A	59	Asp	Asn
		247	C	T			
		512	C	A			
		592	A	G			
9	VP6	30	G	A	5	Met	Ile
		166*	G	A	51	Ala	Thr
		168*	A	G	51	Ala	
		714	G	A			
	NS4	864	G	A			
		969	A	G			
10	NS3	151	G	A			

*mutations present in the same codon

Table 11. Number of sequencing reads mapped to specific segment.

Segment	BTV-8$_{NET2007(blood)}$ n=746,830	BTV-8$_{NET2007(1KC)}$ n=1,013,474	BTV-8$_{NET2007(1KC-1BHK)}$ n=935,152	BTV-8$_{NET2007(1KC-2BHK)}$ n=1,210,870	BTV-8$_{NET2007(1KC)}$ n=1,057,314	BTV-8$_{NET2007(1KC-1BHK)}$ n=1,121,860	BTV-8$_{NET2007(1KC-2BHK)}$ n=1,102,576
1	64,298	202,966	168,436	212,171	175,947	212,148	188,854
2	110,867	142,787	128,157	167,334	167,972	149,994	155,230
3	118,959	141,247	137,284	171,899	162,033	161,714	160,489
4	73,380	98,522	93,542	117,047	100,560	110,123	108,899
5	75,065	92,875	87,426	117,116	100,580	99,530	106,745
6	61,501	90,105	82,903	117,952	96,991	107,884	106,492
7	52,928	67,352	65,517	85,587	63,560	78,113	68,837
8	44,264	62,353	62,332	79,176	62,614	70,209	69,995
9	51,430	57,943	56,312	68,895	64,618	62,467	64,776
10	37,427	47,427	44,144	62,374	51,542	58,018	63,222
Total matched	690,119	1,003,577	926,053	1,199,551	1,046,417	1,110,200	1,093,539
% Matched	92.4	99.0	99.0	99.1	99.0	99.0	99.2

n - total number of reads

Table 12. Number and quality of reads covering detected variants in BTV-$8_{NET2007}$(blood) before and after passage in KC and BHK-21 cells (first replicate).

	BTV-$8_{NET2007(blood)}$				BTV-$8_{NET2007(1KC)}$				BTV-$8_{NET2007(1KC-1BHK)}$				BTV-$8_{NET2007(1KC-2BHK)}$			
SEGMENT	Mean number of reads	Minimum number of reads	Maximum number of reads	Mean read quality (Q)	Mean number of reads	Minimum number of reads	Maximum number of reads	Mean read quality (Q)	Mean number of reads	Minimum number of reads	Maximum number of reads	Mean read quality (Q)	Mean number of reads	Minimum number of reads	Maximum number of reads	Mean read quality (Q)
1	3321.6	2085	6041	36.3	10859.1	6211	19230	37.1	7892.0	5261	16284	36.2	10337.1	5931	20626	36.3
2	7743.6	3161	12762	36.9	10170.0	5022	13059	37.2	7589.1	5310	12595	37.0	10760.0	8385	17571	37.2
3	8687.2	6264	16515	35.8	10403.1	5846	18131	37.1	8351.3	6063	11085	36.6	11464.8	7545	17284	36.6
4	7685.4	5757	12565	37.1	10579.6	7719	17320	37.3	8479.1	6817	10917	36.9	11467.2	8878	19476	36.9
5	8906.6	6064	13940	37.1	11019.2	7102	16625	37.3	8738.6	7072	15677	37.3	12738.3	10320	21743	37.3
6	7772.1	5424	13944	37.0	11612.0	7857	16219	37.1	9033.8	6958	14678	37.4	13430.1	11208	20232	37.4
7	9527.8	6569	16277	36.8	12270.6	8386	19105	37.0	10092.0	6508	20211	37.1	14227.4	9299	26144	37.2
8	8237.8	6028	14337	36.9	11504.7	7467	18551	37.4	9398.1	6649	16267	37.4	12957.1	8818	16625	37.3
9	10126.6	5703	18161	36.4	11439.5	6640	19393	36.4	9911.3	6258	20247	36.7	12535.7	8898	22017	36.8
10	9537.7	6352	15368	37.3	11775.3	8421	18134	37.4	9617.4	7501	16500	37.2	14697.7	12478	25741	37.4
Mean (All)	8154.6	5340.7	13991.0	36.8	11163.3	7067.1	17576.7	37.2	8910.32	6439.70	15446.1	37.0	12461.6	9176.0	20745.9	37.1

Table 13 .Number and quality of reads covering detected variants in BTV-$8_{NET2007(blood)}$ before and after passage in KC and BHK-21 cells (second replicate).

	BTV-$8_{NET2007(1KC)}$				BTV-$8_{NET2007(1KC-1BHK)}$				BTV-$8_{NET2007(1KC-2BHK)}$			
SEGMENT	Mean number of reads	Minimum number of reads	Maximum number of reads	Average read quality (Q)	Mean number of reads	Minimum number of reads	Maximum number of reads	Average read quality (Q)	Mean number of reads	Minimum number of reads	Maximum number of reads	Average read quality (Q)
1	9399.6	5402	17466	37.1	10205.3	6361	18177	36.4	9126.5	5433	21741	36.2
2	11886.7	5654	20989	37.4	9369.4	4942	14138	36.9	9708.3	5926	14439	37.2
3	12101.5	7292	23298	37.3	10281.2	6853	15176	36.1	10517.3	6775	20797	36.6
4	10938.4	7798	19008	37.4	10318.4	8094	12830	37.3	10433.4	8007	14826	36.9
5	11842.1	9180	20833	37.5	10860.5	8904	18228	37.1	11591.8	9105	19708	37.4
6	12221.7	9088	20701	37.4	12467.8	10114	24740	37.1	12452.0	10028	21570	37.4
7	11680.7	6885	20411	37.3	12643.5	8660	15638	36.8	11311.1	7615	20909	36.9
8	11591.3	6975	20321	37.4	11308.0	8279	14346	37.4	11215.8	7567	18319	37.1
9	12813.9	7909	23750	37.1	10942.1	7804	16134	36.4	11679.3	8514	15212	36.0
10	13419.8	9368	20749	37.6	12860.3	11428	14413	37.2	13942.4	12480	15268	37.2
Mean (All)	11789.6	7555.1	20752.6	37.35	11125.6	8143.9	16382.0	36.8	11197.8	8145.0	18278.9	36.9

References

Alexander, K.A., Maclachlan, N.J., Kat, P.W., House, C., Obrien, S.J., Lerche, N.W., Sawyer, M., Frank, L.G., Holekamp, K., Smale, L., Mcnutt, J.W., Laurenson, M.K., Mills, M.G.L., Osburn, B.I., 1994. Evidence of Natural Bluetongue Virus-Infection among African Carnivores. Am J Trop Med Hyg 51, 568-576.

Alexander, R.A., Haig, D.A., Adelaar, T.F., 1947. The attenuation of bluetongue virus by serial passage through fertile eggs. Onderstepoort J Vet Sci Anim Ind 21, 231-241.

Anderson, G.A., Stott, J.L., Gershwin, L.J., Osburn, B.I., 1985. Subclinical and clinical bluetongue disease in cattle: clinical, pathological and pathogenic considerations. Prog Clin Biol Res 178, 103-107.

Anthony, S.J., Maan, S., Maan, N., Kgosana, L., Bachanek-Bankowska, K., Batten, C., Darpel, K.E., Sutton, G., Attoui, H., Mertens, P.P., 2009. Genetic and phylogenetic analysis of the outer-coat proteins VP2 and VP5 of epizootic haemorrhagic disease virus (EHDV): comparison of genetic and serological data to characterise the EHDV serogroup. Virus Res 145, 200-210.

Arnaud, F., Black, S.G., Murphy, L., Griffiths, D.J., Neil, S.J., Spencer, T.E., Palmarini, M., 2010. Interplay between ovine bone marrow stromal cell antigen 2/tetherin and endogenous retroviruses. J Virol 84, 4415-4425.

Attoui, H., Mendez-Lopez, M.R., Rao, S., Hurtado-Alendes, A., Lizaraso-Caparo, F., Jaafar, F.M., Samuel, A.R., Belhouchet, M., Pritchard, L.I., Melville, L., Weir, R.P., Hyatt, A.D., Davis, S.S., Lunt, R., Calisher, C.H., Tesh, R.B., Fujita, R., Mertens, P.P., 2009. Peruvian horse sickness virus and Yunnan orbivirus, isolated from vertebrates and mosquitoes in Peru and Australia. Virology 394, 298-310.

Backx, A., Heutink, R., van Rooij, E., van Rijn, P., 2009. Transplacental and oral transmission of wild-type bluetongue virus serotype 8 in cattle after experimental infection. Vet Microbiol 138, 235-243.

Bansal, O.B., Stokes, A., Bansal, A., Bishop, D., Roy, P., 1998. Membrane organization of bluetongue virus nonstructural glycoprotein NS3. J Virol 72, 3362-3369.

Baranowski, E., Sevilla, N., Verdaguer, N., Ruiz-Jarabo, C.M., Beck, E., Domingo, E., 1998. Multiple virulence determinants of foot-and-mouth disease virus in cell culture. J Virol 72, 6362-6372.

Barratt-Boyes, S.M., MacLachlan, N.J., 1994. Dynamics of viral spread in bluetongue virus infected calves. Vet Microbiol 40, 361-371.

Barratt-Boyes, S.M., Rossitto, P.V., Taylor, B.C., Ellis, J.A., MacLachlan, N.J., 1995. Response of the regional lymph node to bluetongue virus infection in calves. Vet Immunol Immunopathol 45, 73-84.

Basak, A.K., Grimes, J.M., Gouet, P., Roy, P., Stuart, D.I., 1997. Structures of orbivirus VP7: implications for the role of this protein in the viral life cycle. Structure 5, 871-883.

Basak, A.K., Stuart, D.I., Roy, P., 1992. Preliminary crystallographic study of bluetongue virus capsid protein, VP7. J Mol Biol 228, 687-689.

Batten, C., Darpel, K., Henstock, M., Fay, P., Veronesi, E., Gubbins, S., Graves, S., Frost, L., Oura, C., 2014. Evidence for transmission of bluetongue virus serotype 26 through direct contact. PLoS One 9, e96049.

Batten, C.A., Bachanek-Bankowska, K., Bin-Tarif, A., Kgosana, L., Swain, A.J., Corteyn, M., Darpel, K., Mellor, P.S., Elliott, H.G., Oura, C.A., 2008a. Bluetongue virus: European Community inter-laboratory comparison tests to evaluate ELISA and RT-PCR detection methods. Vet Microbiol 129, 80-88.

Batten, C.A., Edwards, L., Bin-Tarif, A., Henstock, M.R., Oura, C.A., 2011. Infection kinetics of Epizootic Haemorrhagic Disease virus serotype 6 in Holstein-Friesian cattle. Vet Microbiol 154, 23-28.

Batten, C.A., Maan, S., Shaw, A.E., Maan, N.S., Mertens, P.P., 2008b. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. Virus Res 137, 56-63.

Baylis, M., O'Connell, L., Mellor, P.S., 2008. Rates of bluetongue virus transmission between Culicoides sonorensis and sheep. Med Vet Entomol 22, 228-237.

Beaton, A.R., Rodriguez, J., Reddy, Y.K., Roy, P., 2002. The membrane trafficking protein calpactin forms a complex with bluetongue virus protein NS3 and mediates virus release. Proc Natl Acad Sci U S A 99, 13154-13159.

Bekker, J.G., De Kock, G.W., Quinlan, J.B., 1934. The occurance and identification of Bluetongue in cattle - the so-called Pseudo-Foot and Mouth Disease in South Africa. Onderstepoort J Vet Sci Anim Ind 2, 393-449.

Belaganahalli, M.N., Maan, S., Maan, N.S., Brownlie, J., Tesh, R., Attoui, H., Mertens, P.P., 2015. Genetic characterization of the tick-borne orbiviruses. Viruses 7, 2185-2209.

Belaganahalli, M.N., Maan, S., Maan, N.S., Nomikou, K., Guimera, M., Brownlie, J., Tesh, R., Attoui, H., Mertens, P.P., 2013. Full genome sequencing of Corriparta virus, identifies California mosquito pool virus as a member of the Corriparta virus species. PLoS One 8, e70779.

Belaganahalli, M.N., Maan, S., Maan, N.S., Pritchard, I., Kirkland, P.D., Brownlie, J., Attoui, H., Mertens, P.P., 2014. Full genome characterization of the culicoides-borne marsupial orbiviruses: Wallal virus, Mudjinbarry virus and Warrego viruses. PLoS One 9, e108379.

Belhouchet, M., Mohd Jaafar, F., Firth, A.E., Grimes, J.M., Mertens, P.P., Attoui, H., 2011. Detection of a fourth orbivirus non-structural protein. PLoS One 6, e25697.

Bernard, K.A., Israel, B.A., Thompson, L.H., Homan, E.J., Schultz, K.T., 1994. Virulence-associated antigenic and genetic characteristics of bluetongue virus-17 isolates. Virology 201, 321-329.

Bernard, K.A., Klimstra, W.B., Johnston, R.E., 2000. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology 276, 93-103.

Bhattacharya, B., Celma, C.C., Roy, P., 2015. Influence of cellular trafficking pathway on bluetongue virus infection in ovine cells. Viruses 7, 2378-2403.

Bhattacharya, B., Noad, R.J., Roy, P., 2007. Interaction between Bluetongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. Virol J 4, 7.

Bhattacharya, B., Roy, P., 2008. Bluetongue virus outer capsid protein VP5 interacts with membrane lipid rafts via a SNARE domain. J Virol 82, 10600-10612.

Bonneau, K.R., DeMaula, C.D., Mullens, B.A., MacLachlan, N.J., 2002. Duration of viraemia infectious to Culicoides sonorensis in bluetongue virus-infected cattle and sheep. Vet Microbiol 88, 115-125.

Bonneau, K.R., MacLachlan, N.J., 2004. Genetic diversification of field strains of bluetongue virus. Vet Ital 40, 446-447.

Bonneau, K.R., Mullens, B.A., MacLachlan, N.J., 2001. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and Culicoides sonorensis. J Virol 75, 8298-8305.

Bonneau, K.R., Zhang, N., Zhu, J., Zhang, F., Li, Z., Zhang, K., Xiao, L., Xiang, W., MacLachlan, N.J., 1999. Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. Virus Res 61, 153-160.

Boone, J.D., Balasuriya, U.B., Karaca, K., Audonnet, J.C., Yao, J., He, L., Nordgren, R., Monaco, F., Savini, G., Gardner, I.A., Maclachlan, N.J., 2007. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. Vaccine 25, 672-678.

Borden, E.C., Shope, R.E., Murphy, F.A., 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus--a new taxonomic group. Physiocochemical and serological studies. J Gen Virol 13, 261-271.

Boyce, M., Celma, C.C., Roy, P., 2008. Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. J Virol 82, 8339-8348.

Boyce, M., Celma, C.C., Roy, P., 2012. Bluetongue virus non-structural protein 1 is a positive regulator of viral protein synthesis. Virol J 9, 178.

Boyce, M., Wehrfritz, J., Noad, R., Roy, P., 2004. Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity. J Virol 78, 3994-4002.

Brenner, J., Batten, C., Yadin, H., Bumbarov, V., Friedgut, O., Rotenberg, D., Golender, N., Oura, C.A., 2011. Clinical syndromes associated with the circulation of multiple serotypes of bluetongue virus in dairy cattle in Israel. Vet Rec 169, 389.

Brenner, J., Oura, C., Asis, I., Maan, S., Elad, D., Maan, N., Friedgut, O., Nomikou, K., Rotenberg, D., Bumbarov, V., Mertens, P., Yadin, H., Batten, C., 2010. Multiple serotypes of bluetongue virus in sheep and cattle, Israel. Emerg Infect Dis 16, 2003-2004.

Brewer, A.W., Osburn, B.I., 1998. Sequential distribution of neurovirulent and avirulent strains of bluetongue virus in neonatal mice by RT-PCR. Arch Virol 143, 145-155.

Brookes, S.M., Hyatt, A.D., Eaton, B.T., 1993. Characterization of virus inclusion bodies in bluetongue virus-infected cells. J Gen Virol 74 (Pt 3), 525-530.

Brown, S.E., Morrison, H.G., Karabatsos, N., Knudson, D.L., 1991. Genetic relatedness of two new Orbivirus serogroups: Orungo and Lebombo. J Gen Virol 72 (Pt 5), 1065-1072.

Burkhardt, C., Sung, P.Y., Celma, C.C., Roy, P., 2014. Structural constraints in the packaging of bluetongue virus genomic segments. J Gen Virol 95, 2240-2250.

Byrnes, A.P., Griffin, D.E., 1998. Binding of Sindbis virus to cell surface heparan sulfate. J Virol 72, 7349-7356.

Byrnes, A.P., Griffin, D.E., 2000. Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. J Virol 74, 644-651.

Calvo-Pinilla, E., Navasa, N., Anguita, J., Ortego, J., 2012. Multiserotype protection elicited by a combinatorial prime-boost vaccination strategy against bluetongue virus. PLoS One 7, e34735.

Calvo-Pinilla, E., Nieto, J.M., Ortego, J., 2010. Experimental oral infection of bluetongue virus serotype 8 in type I interferon receptor-deficient mice. J Gen Virol 91, 2821-2825.

Calvo-Pinilla, E., Rodriguez-Calvo, T., Anguita, J., Sevilla, N., Ortego, J., 2009a. Establishment of a bluetongue virus infection model in mice that are deficient in the alpha/beta interferon receptor. PLoS One 4, e5171.

Calvo-Pinilla, E., Rodriguez-Calvo, T., Sevilla, N., Ortego, J., 2009b. Heterologous prime boost vaccination with DNA and recombinant modified vaccinia virus Ankara protects IFNAR(-/-) mice against lethal bluetongue infection. Vaccine 28, 437-445.

Caporale, M., Di Gialleonorado, L., Janowicz, A., Wilkie, G., Shaw, A., Savini, G., Van Rijn, P.A., Mertens, P., Di Ventura, M., Palmarini, M., 2014. Virus and host factors affecting the clinical outcome of bluetongue virus infection. J Virol 88, 10399-10411.

Caporale, M., Wash, R., Pini, A., Savini, G., Franchi, P., Golder, M., Patterson-Kane, J., Mertens, P., Di Gialleonardo, L., Armillotta, G., Lelli, R., Kellam, P., Palmarini, M., 2011. Determinants of bluetongue virus virulence in murine models of disease. J Virol 85, 11479-11489.

Caporale, V., Giovannini, A., 2010. Bluetongue control strategy, including recourse to vaccine: a critical review. Rev Sci Tech 29, 573-591.

Caracappa, S., Torina, A., Guercio, A., Vitale, F., Calabro, A., Purpari, G., Ferrantelli, V., Vitale, M., Mellor, P.S., 2003. Identification of a novel bluetongue virus vector species of Culicoides in Sicily. Vet Rec 153, 71-74.

Carpi, G., Holmes, E.C., Kitchen, A., 2010. The evolutionary dynamics of bluetongue virus. J Mol Evol 70, 583-592.

Carr, M.A., Brewer, A.W., Osburn, B.I., 1995. Growth of a neuroinvasive strain of bluetongue virus in suckling mice. Arch Virol 140, 915-925.

Carr, M.A., de Mattos, C.C., de Mattos, C.A., Osburn, B.I., 1994. Association of bluetongue virus gene segment 5 with neuroinvasiveness. J Virol 68, 1255-1257.

Casadevall, A., Pirofski, L., 2001. Host-pathogen interactions: the attributes of virulence. The J Infect Dis 184, 337-344.

Celma, C.C., Bhattacharya, B., Eschbaumer, M., Wernike, K., Beer, M., Roy, P., 2014. Pathogenicity study in sheep using reverse-genetics-based reassortant bluetongue viruses. Vet Microbiol 174, 139-147.

Celma, C.C., Boyce, M., van Rijn, P.A., Eschbaumer, M., Wernike, K., Hoffmann, B., Beer, M., Haegeman, A., De Clercq, K., Roy, P., 2013. Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. J Virol 87, 9856-9864.

Celma, C.C., Roy, P., 2009. A viral nonstructural protein regulates bluetongue virus trafficking and release. J Virol 83, 6806-6816.

Chauveau, E., Doceul, V., Lara, E., Adam, M., Breard, E., Sailleau, C., Viarouge, C., Desprat, A., Meyer, G., Schwartz-Cornil, I., Ruscanu, S., Charley, B., Zientara, S., Vitour, D., 2012. Sensing and control of bluetongue virus infection in epithelial cells via RIG-I and MDA5 helicases. J Virol 86, 11789-11799.

Chauveau, E., Doceul, V., Lara, E., Breard, E., Sailleau, C., Vidalain, P.O., Meurs, E.F., Dabo, S., Schwartz-Cornil, I., Zientara, S., Vitour, D., 2013. NS3 of bluetongue virus interferes with the induction of type I interferon. J Virol 87, 8241-8246.

Chen, L.M., Davis, C.T., Zhou, H., Cox, N.J., Donis, R.O., 2008. Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. PLoS Pathog 4, e1000072.

Chen, S., Short, J.A., Young, D.F., Killip, M.J., Schneider, M., Goodbourn, S., Randall, R.E., 2010. Heterocellular induction of interferon by negative-sense RNA viruses. Virology 407, 247-255.

Cheng, Y., Austin, S.C., Rocca, B., Koller, B.H., Coffman, T.M., Grosser, T., Lawson, J.A., FitzGerald, G.A., 2002. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science 296, 539-541.

Chiang, E.T., Persaud-Sawin, D.A., Kulkarni, S., Garcia, J.G., Imani, F., 2006. Bluetongue virus and double-stranded RNA increase human vascular permeability: role of p38 MAPK. J Clin Immunol 26, 406-416.

Chong, Y.L., Padhi, A., Hudson, P.J., Poss, M., 2010. The effect of vaccination on the evolution and population dynamics of avian paramyxovirus-1. PLoS Pathog 6, e1000872.

Clarke, D.K., Duarte, E.A., Moya, A., Elena, S.F., Domingo, E., Holland, J., 1993. Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. J Virol 67, 222-228.

Coetzee, P., Stokstad, M., Venter, E.H., Myrmel, M., Van Vuuren, M., 2012a. Bluetongue: a historical and epidemiological perspective with the emphasis on South Africa. Virol J 9, 198.

Coetzee, P., Van Vuuren, M., Stokstad, M., Myrmel, M., van Gennip, R.G., van Rijn, P.A., Venter, E.H., 2014. Viral replication kinetics and in vitro cytopathogenicity of parental and reassortant strains of bluetongue virus serotype 1, 6 and 8. Vet Microbiol 171, 53-65.

Coetzee, P., Van Vuuren, M., Stokstad, M., Myrmel, M., Venter, E.H., 2012b. Bluetongue virus genetic and phenotypic diversity: towards identifying the molecular determinants that influence virulence and transmission potential. Vet Microbiol 161, 1-12.

Coffey, L.L., Vignuzzi, M., 2011. Host alternation of chikungunya virus increases fitness while restricting population diversity and adaptability to novel selective pressures. J Virol 85, 1025-1035.

Conraths, F.J., Gethmann, J.M., Staubach, C., Mettenleiter, T.C., Beer, M., Hoffmann, B., 2009. Epidemiology of bluetongue virus serotype 8, Germany. Emerg Infect Dis 15, 433-435.

Cowley, J.A., Gorman, B.M., 1987. Genetic reassortants for identification of the genome segment coding for the bluetongue virus hemagglutinin. J Virol 61, 2304-2306.

Cox, H.R., 1954. Bluetongue. Bacteriol Rev 18, 239-253.

Daffis, S., Szretter, K.J., Schriewer, J., Li, J., Youn, S., Errett, J., Lin, T.Y., Schneller, S., Zust, R., Dong, H., Thiel, V., Sen, G.C., Fensterl, V., Klimstra, W.B., Pierson, T.C., Buller, R.M., Gale, M., Jr., Shi, P.Y., Diamond, M.S., 2010. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature 468, 452-456.

Dal Pozzo, F., Saegerman, C., Thiry, E., 2009. Bovine infection with bluetongue virus with special emphasis on European serotype 8. Vet J 182, 142-151.

Darpel, K.E., Batten, C.A., Veronesi, E., Shaw, A.E., Anthony, S., Bachanek-Bankowska, K., Kgosana, L., bin-Tarif, A., Carpenter, S., Muller-Doblies, U.U., Takamatsu, H.H., Mellor, P.S., Mertens, P.P., Oura, C.A., 2007. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. Vet Rec 161, 253-261.

Darpel, K.E., Monaghan, P., Simpson, J., Anthony, S.J., Veronesi, E., Brooks, H.W., Elliott, H., Brownlie, J., Takamatsu, H.H., Mellor, P.S., Mertens, P.P., 2012. Involvement of the skin during bluetongue virus infection and replication in the ruminant host. Vet Res 43, 40.

De Clercq, K., Mertens, P., De Leeuw, I., Oura, C., Houdart, P., Potgieter, A.C., Maan, S., Hooyberghs, J., Batten, C., Vandemeulebroucke, E., Wright, I.M., Maan, N., Riocreux, F., Sanders, A., Vanderstede, Y., Nomikou, K., Raemaekers, M., Bin-Tarif, A., Shaw, A., Henstock, M., Breard, E., Dubois, E., Gastaldi-Thiery, C., Zientara, S., Verheyden, B., Vandenbussche, F., 2009. Emergence of bluetongue serotypes in Europe, part 2: the occurrence of a BTV-11 strain in Belgium. Transbound Emerg Dis 56, 355-361.

DeMaula, C.D., Jutila, M.A., Wilson, D.W., MacLachlan, N.J., 2001. Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. J Gen Virol 82, 787-794.

DeMaula, C.D., Leutenegger, C.M., Bonneau, K.R., MacLachlan, N.J., 2002a. The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue. Virology 296, 330-337.

DeMaula, C.D., Leutenegger, C.M., Jutila, M.A., MacLachlan, N.J., 2002b. Bluetongue virus-induced activation of primary bovine lung microvascular endothelial cells. Vet Immunol Immunopath 86, 147-157.

Diprose, J.M., Burroughs, J.N., Sutton, G.C., Goldsmith, A., Gouet, P., Malby, R., Overton, I., Zientara, S., Mertens, P.P., Stuart, D.I., Grimes, J.M., 2001. Translocation portals for the substrates and products of a viral transcription complex: the bluetongue virus core. EMBO J 20, 7229-7239.

Diprose, J.M., Grimes, J.M., Sutton, G.C., Burroughs, J.N., Meyer, A., Maan, S., Mertens, P.P., Stuart, D.I., 2002. The core of bluetongue virus binds double-stranded RNA. J Virol 76, 9533-9536.

Doceul, V., Chauveau, E., Lara, E., Breard, E., Sailleau, C., Zientara, S., Vitour, D., 2014. Dual modulation of type I interferon response by bluetongue virus. J Virol 88, 10792-10802.

Dortmans, J.C., Koch, G., Rottier, P.J., Peeters, B.P., 2011. Virulence of Newcastle disease virus: what is known so far? Vet Res 42, 122.

du Toit, P.J., 1944. Transmission of Blue-Tongue and Horse-Sickness by Culicoides. Onderstepoort J Vet Res 19, 7-16.

Dungu, B., Gerdes, T., Smit, T., 2004. The use of vaccination in the control of bluetongue in southern Africa. Vet Ital 40, 616-622.

Eaton, B.T., Crameri, G.S., 1989. The site of bluetongue virus attachment to glycophorins from a number of animal erythrocytes. J Gen Virol 70 (Pt 12), 3347-3353.

Eaton, B.T., Hyatt, A.D., Brookes, S.M., 1990. The replication of bluetongue virus. Curr Top Microbiol Immunol 162, 89-118.

Ebel, G.D., Fitzpatrick, K.A., Lim, P.Y., Bennett, C.J., Deardorff, E.R., Jerzak, G.V., Kramer, L.D., Zhou, Y., Shi, P.Y., Bernard, K.A., 2011. Nonconsensus West Nile virus genomes arising during mosquito infection suppress pathogenesis and modulate virus fitness in vivo. J Virol 85, 12605-12613.

Elbers, A., Backx, A., van der Spek, A., Ekker, M., Leijs, P., Steijn, K., van Langen, H., van Rijn, P., 2008a. [Epidemiology of bluetongue virus serotype 8 outbreaks in the Netherlands in 2006]. Tijdschr Diergeneeskd 133, 222-229.

Elbers, A.R., Backx, A., Ekker, H.M., van der Spek, A.N., van Rijn, P.A., 2008b. Performance of clinical signs to detect bluetongue virus serotype 8 outbreaks in cattle and sheep during the 2006-epidemic in The Netherlands. Vet Microbiol 129, 156-162.

Elbers, A.R., Backx, A., Mintiens, K., Gerbier, G., Staubach, C., Hendrickx, G., van der Spek, A., 2008c. Field observations during the Bluetongue serotype 8 epidemic in 2006. II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. Prev Vet Med 87, 31-40.

Elbers, A.R., van der Spek, A.N., van Rijn, P.A., 2009. Epidemiologic characteristics of bluetongue virus serotype 8 laboratory-confirmed outbreaks in The Netherlands in 2007 and a comparison with the situation in 2006. Prev Vet Med 92, 1-8.

Erasmus, B.J., 1975. Bluetongue in sheep and goats. Aust Vet J 51, 165-170.

Erasmus, B.J., 1985. The history of bluetongue. Prog Clin Biol Res 178, 7-12.

Eschbaumer, M., Hoffmann, B., Konig, P., Teifke, J.P., Gethmann, J.M., Conraths, F.J., Probst, C., Mettenleiter, T.C., Beer, M., 2009. Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep. Vaccine 27, 4169-4175.

Eschbaumer, M., Hoffmann, B., Moss, A., Savini, G., Leone, A., Konig, P., Zemke, J., Conraths, F., Beer, M., 2010. Emergence of bluetongue virus serotype 6 in Europe--German field data and experimental infection of cattle. Vet Microbiol 143, 189-195.

Eschbaumer, M., Schulz, C., Wackerlin, R., Gauly, M., Beer, M., Hoffmann, B., 2011. Limitations of sandwich ELISAs for bluetongue virus antibody detection. Vet Rec 168, 643.

Farci, P., Strazzera, R., Alter, H.J., Farci, S., Degioannis, D., Coiana, A., Peddis, G., Usai, F., Serra, G., Chessa, L., Diaz, G., Balestrieri, A., Purcell, R.H., 2002. Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. Proc Natl Acad Sci U S A 99, 3081-3086.

Feenstra, F., Pap, J.S., van Rijn, P.A., 2015. Application of bluetongue Disabled Infectious Single Animal (DISA) vaccine for different serotypes by VP2 exchange or incorporation of chimeric VP2. Vaccine 33, 812-818.

Feenstra, F., van Gennip, R.G., van de Water, S.G., van Rijn, P.A., 2014. RNA elements in open reading frames of the bluetongue virus genome are essential for virus replication. PLoS One 9, e92377.

Feng, N., Yasukawa, L.L., Sen, A., Greenberg, H.B., 2013. Permissive replication of homologous murine rotavirus in the mouse intestine is primarily regulated by VP4 and NSP1. J Virol 87, 8307-8316.

Fillmore, G.C., Lin, H., Li, J.K., 2002. Localization of the single-stranded RNA-binding domains of bluetongue virus nonstructural protein NS2. J Virol 76, 499-506.

Firth, A.E., 2008. Bioinformatic analysis suggests that the Orbivirus VP6 cistron encodes an overlapping gene. Virol J 5, 48.

Forzan, M., Marsh, M., Roy, P., 2007. Bluetongue virus entry into cells. J Virol 81, 4819-4827.

Forzan, M., Wirblich, C., Roy, P., 2004. A capsid protein of nonenveloped Bluetongue virus exhibits membrane fusion activity. Proc Natl Acad Sci U S A 101, 2100-2105.

Foster, N.M., Luedke, A.J., Parsonson, I.M., Walton, T.E., 1991. Temporal relationships of viremia, interferon activity, and antibody responses of sheep infected with several bluetongue virus strains. Am J Vet Res 52, 192-196.

Franchi, P., Mercante, M.T., Ronchi, G.F., Armillotta, G., Ulisse, S., Molini, U., Di Ventura, M., Lelli, R., Savini, G., Pini, A., 2008. Laboratory tests for evaluating the level of attenuation of bluetongue virus. J Virol Methods 153, 263-265.

Freed, E.O., 2002. Viral late domains. J Virol 76, 4679-4687.

Froissart, R., Wilke, C.O., Montville, R., Remold, S.K., Chao, L., Turner, P.E., 2004. Co-infection weakens selection against epistatic mutations in RNA viruses. Genetics 168, 9-19.

Fu, H., Leake, C.J., Mertens, P.P., Mellor, P.S., 1999. The barriers to bluetongue virus infection, dissemination and transmission in the vector, Culicoides variipennis (Diptera: Ceratopogonidae). Arch Virol 144, 747-761.

Fulton, R.W., Pearson, N.J., 1982. Interferon induction in bovine and feline monolayer cultures by four bluetongue virus serotypes. Can J Comp Med 46, 100-102.

Gale, P., Brouwer, A., Ramnial, V., Kelly, L., Kosmider, R., Fooks, A.R., Snary, E.L., 2010. Assessing the impact of climate change on vector-borne viruses in the EU through the elicitation of expert opinion. Epidemiol Infect 138, 214-225.

Garcia-Sastre, A., 2011. 2 methylate or not 2 methylate: viral evasion of the type I interferon response. Nat Immunol 12, 114-115.

Gardner, C.L., Hritz, J., Sun, C., Vanlandingham, D.L., Song, T.Y., Ghedin, E., Higgs, S., Klimstra, W.B., Ryman, K.D., 2014. Deliberate attenuation of chikungunya virus by adaptation to heparan sulfate-dependent infectivity: a model for rational arboviral vaccine design. PLoS Neg Trop Dis s 8, e2719.

Ghalib, H.W., Cherrington, J.M., Osburn, B.I., 1985. Virological, clinical and serological responses of sheep infected with tissue culture adapted bluetongue virus serotypes 10, 11, 13 and 17. Vet Microbiol 10, 179-188.

Gibbs, E.P., Greiner, E.C., 1994. The epidemiology of bluetongue. Comp Immunol Microbiol Infect Dis 17, 207-220.

Gibbs, E.P., Lawman, M.J., Herniman, K.A., 1979. Preliminary observations on transplacental infection of bluetongue virus in sheep-a possible overwintering mechanism. Res Vet Sci 27, 118-120.

Goldsmit, L., Barzilai, E., Tadmor, A., 1975. Comparative Sensitivity of Sheep and Chicken Embryos to Bluetongue Virus and Observations on Viremia in Experimentally Infected Sheep. Aus Vet J 51, 190-196.

Gouet, P., Diprose, J.M., Grimes, J.M., Malby, R., Burroughs, J.N., Zientara, S., Stuart, D.I., Mertens, P.P., 1999. The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. Cell 97, 481-490.

Gould, A.R., Eaton, B.T., 1990. The amino acid sequence of the outer coat protein VP2 of neutralizing monoclonal antibody-resistant, virulent and attenuated bluetongue viruses. Virus Res 17, 161-172.

Gould, A.R., Hyatt, A.D., Eaton, B.T., 1988. Morphogenesis of a bluetongue virus variant with an amino acid alteration at a neutralization site in the outer coat protein, VP2. Virology 165, 23-32.

Grimes, J., Basak, A.K., Roy, P., Stuart, D., 1995. The crystal structure of bluetongue virus VP7. Nature 373, 167-170.

Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Zientara, S., Mertens, P.P., Stuart, D.I., 1998. The atomic structure of the bluetongue virus core. Nature 395, 470-478.

Grimes, J.M., Jakana, J., Ghosh, M., Basak, A.K., Roy, P., Chiu, W., Stuart, D.I., Prasad, B.V., 1997. An atomic model of the outer layer of the bluetongue virus core derived from X-ray crystallography and electron cryomicroscopy. Structure 5, 885-893.

Guirakhoo, F., Catalan, J.A., Monath, T.P., 1995. Adaptation of bluetongue virus in mosquito cells results in overexpression of NS3 proteins and release of virus particles. Arch Virol 140, 967-974.

Haller, B.L., Barkon, M.L., Vogler, G.P., Virgin, H.W.t., 1995. Genetic mapping of reovirus virulence and organ tropism in severe combined immunodeficient mice: organ-specific virulence genes. J Virol 69, 357-364.

Hamers, C., Rehbein, S., Hudelet, P., Blanchet, M., Lapostolle, B., Cariou, C., Duboeuf, M., Goutebroze, S., 2009. Protective duration of immunity of an inactivated bluetongue (BTV) serotype 2 vaccine against a virulent BTV serotype 2 challenge in sheep. Vaccine 27, 2789-2793.

Hassan, S.H., Wirblich, C., Forzan, M., Roy, P., 2001. Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. J Virol 75, 8356-8367.

Hassan, S.S., Roy, P., 1999. Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. J Virol 73, 9832-9842.

Hayama, E., Li, J.K., 1994. Mapping and characterization of antigenic epitopes and the nucleic acid-binding domains of the VP6 protein of bluetongue viruses. J Virol 68, 3604-3611.

He, C.Q., Ding, N.Z., He, M., Li, S.N., Wang, X.M., He, H.B., Liu, X.F., Guo, H.S., 2010. Intragenic recombination as a mechanism of genetic diversity in bluetongue virus. J Virol 84, 11487-11495.

Hemati, B., Contreras, V., Urien, C., Bonneau, M., Takamatsu, H.H., Mertens, P.P., Breard, E., Sailleau, C., Zientara, S., Schwartz-Cornil, I., 2009. Bluetongue virus targets conventional dendritic cells in skin lymph. J Virol 83, 8789-8799.

Hewat, E.A., Booth, T.F., Roy, P., 1992a. Structure of bluetongue virus particles by cryoelectron microscopy. J Struct Biol 109, 61-69.

Hewat, E.A., Booth, T.F., Wade, R.H., Roy, P., 1992b. 3-D reconstruction of bluetongue virus tubules using cryoelectron microscopy. J Struct Biol 108, 35-48.

Hoffmann, B., Eschbaumer, M., Beer, M., 2009. Real-time quantitative reverse transcription-PCR assays specifically detecting bluetongue virus serotypes 1, 6, and 8. J Clin Microbiol 47, 2992-2994.

Hofmann, M.A., Renzullo, S., Mader, M., Chaignat, V., Worwa, G., Thuer, B., 2008. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. Emerg Infect Dis 14, 1855-1861.

Hooper, P.T., Lunt, R.A., Stanislawek, W.L., 1996. A trial comparing the virulence of some South African and Australian bluetongue viruses. Aust Vet J 73, 36-37.

Hoshino, Y., Saif, L.J., Kang, S.Y., Sereno, M.M., Chen, W.K., Kapikian, A.Z., 1995. Identification of group A rotavirus genes associated with virulence of a porcine rotavirus and host range restriction of a human rotavirus in the gnotobiotic piglet model. Virology 209, 274-280.

Hourrigan, J.L., Klingsporn, A.L., 1975a. Bluetongue: the disease in cattle. Aust Vet J 51, 170-174.

Hourrigan, J.L., Klingsporn, A.L., 1975b. Epizootiology of bluetongue: the situation in the United States of America. Aust Vet J 51, 203-208.

Howell, P.G., 1960. A preliminary antigenic classification of strains of bluetongue virus. Ondersterpoort J Vet Res 28, 357-363.

Howell, P.G., 1962. The isolation and identification of further antigenic types of African horsesickness virus. Onderstepoort J Vet Res 29, 139:149.

Howell, P.G., Groenewald, D., Visage, C.W., Bosman, A.M., Coetzer, J.A., Guthrie, A.J., 2002. The classification of seven serotypes of equine encephalosis virus and the prevalence of homologous antibody in horses in South Africa. Onderstepoort J Vet Res 69, 79-93.

Howell, P.G., Kumm, N.A., Botha, M.J., 1970. The application of improved techniques to the identification of strains of bluetongue virus. Onderstepoort J Vet Res 37, 59-66.

Huismans, H., 1969. Bluetongue virus-induced interferon synthesis. Onderstepoort J Vet Res 36, 181-185.

Huismans, H., 1979. Protein synthesis in bluetongue virus-infected cells. Virology 92, 385-396.

Huismans, H., Els, H.J., 1979. Characterization of the tubules associated with the replication of three different orbiviruses. Virology 92, 397-406.

Huismans, H., Erasmus, B.J., 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. Onderstepoort J Vet Res 48, 51-58.

Huismans, H., van der Walt, N.T., Cloete, M., Erasmus, B.J., 1987a. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. Virology 157, 172-179.

Huismans, H., van Dijk, A.A., Bauskin, A.R., 1987b. In vitro phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. J Virol 61, 3589-3595.

Hutcheon, D., 1881. Fever of epizootic catarrh. Rept Coll Vet Surg.

Hyatt, A.D., Eaton, B.T., Brookes, S.M., 1989. The release of bluetongue virus from infected cells and their superinfection by progeny virus. Virology 173, 21-34.

Ingham, R.J., Gish, G., Pawson, T., 2004. The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene 23, 1972-1984.

Jameson, P., Grossberg, S.E., 1979. Production of interferon by human tumor cell lines. Arch Virol 62, 209-219.

Jameson, P., Grossberg, S.E., 1981. Induction of interferon with bluetongue virus in various cells. Method Enzymol 78, 254-258.

Jameson, P., Schoenherr, C.K., Grossberg, S.E., 1978. Bluetongue virus, an exceptionally potent interferon inducer in mice. Infect Immun 20, 321-323.

Janowicz, A., Caporale, M., Shaw, A., Gulletta, S., Di Gialleonardo, L., Ratinier, M., Palmarini, M., 2015. Multiple genome segments determine virulence of bluetongue virus serotype 8. J Virol 89, 5238-5249.

Jeggo, M.H., Gumm, I.D., Taylor, W.P., 1983. Clinical and serological response of sheep to serial challenge with different bluetongue virus types. Res Vet Sci 34, 205-211.

Jeggo, M.J., Corteyn, A.H., Taylor, W.P., Davidson, W.L., Gorman, B.M., 1987. Virulence of bluetongue virus for British sheep. Res Vet Sci 42, 24-28.

Jenckel, M., Breard, E., Schulz, C., Sailleau, C., Viarouge, C., Hoffmann, B., Hoper, D., Beer, M., Zientara, S., 2015. Complete coding genome sequence of putative novel bluetongue virus serotype 27. Genom Announ 3.

Jerzak, G., Bernard, K.A., Kramer, L.D., Ebel, G.D., 2005. Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. J Gen Virol 86, 2175-2183.

Jerzak, G.V., Bernard, K., Kramer, L.D., Shi, P.Y., Ebel, G.D., 2007. The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. Virology 360, 469-476.

Jerzak, G.V., Brown, I., Shi, P.Y., Kramer, L.D., Ebel, G.D., 2008. Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. Virology 374, 256-260.

Jones, R.H., Foster, N.M., 1974. Oral infection of Culicoides variipennis with bluetongue virus: development of susceptible and resistant lines from a colony population. J Med Entomol 11, 316-323.

Kar, A.K., Bhattacharya, B., Roy, P., 2007. Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. BMC Mol Biol 8, 4.

Kar, A.K., Ghosh, M., Roy, P., 2004. Mapping the assembly pathway of Bluetongue virus scaffolding protein VP3. Virology 324, 387-399.

Kar, A.K., Iwatani, N., Roy, P., 2005. Assembly and intracellular localization of the bluetongue virus core protein VP3. J Virol 79, 11487-11495.

Kar, A.K., Roy, P., 2003. Defining the structure-function relationships of bluetongue virus helicase protein VP6. J Virol 77, 11347-11356.

Keller, B.C., Fredericksen, B.L., Samuel, M.A., Mock, R.E., Mason, P.W., Diamond, M.S., Gale, M., Jr., 2006. Resistance to alpha/beta interferon is a determinant of West Nile virus replication fitness and virulence. J Virol 80, 9424-9434.

Kingsolver, M.B., Huang, Z., Hardy, R.W., 2013. Insect antiviral innate immunity: pathways, effectors, and connections. J Mol Biol 425, 4921-4936.

Klimstra, W.B., Ryman, K.D., Johnston, R.E., 1998. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. J Virol 72, 7357-7366.

Koumbati, M., Mangana, O., Nomikou, K., Mellor, P.S., Papadopoulos, O., 1999. Duration of bluetongue viraemia and serological responses in experimentally infected European breeds of sheep and goats. Vet Microbiol 64, 277-285.

Laegreid, W.W., Skowronek, A., Stone-Marschat, M., Burrage, T., 1993. Characterization of virulence variants of African horsesickness virus. Virology 195, 836-839.

Lager, I.A., 2004. Bluetongue virus in South America: overview of viruses, vectors, surveillance and unique features. Vet Ital 40, 89-93.

Lauring, A.S., Andino, R., 2010. Quasispecies theory and the behavior of RNA viruses. PLoS Pathog 6, e1001005.

Lauring, A.S., Frydman, J., Andino, R., 2013. The role of mutational robustness in RNA virus evolution. Nat Rev Microbiol 11, 327-336.

Le Blois, H., French, T., Mertens, P.P., Burroughs, J.N., Roy, P., 1992. The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of the virus. Virology 189, 757-761.

Lee, E., Wright, P.J., Davidson, A., Lobigs, M., 2006. Virulence attenuation of Dengue virus due to augmented glycosaminoglycan-binding affinity and restriction in extraneural dissemination. J Gen Virol 87, 2791-2801.

Li, H., Ruan, J., Durbin, R., 2008. Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res 18, 1851-1858.

Libikova, H., Heinz, F., Ujhazyova, D., Stunzner, D., 1978. Orbiviruses of the Kemerovo complex and neurological diseases. Med Microbiol Immunol 166, 255-263.

Liu, Y., Ma, T., Liu, J., Zhao, X., Cheng, Z., Guo, H., Xu, R., Wang, S., 2014. Circulating type 1 vaccine-derived poliovirus may evolve under the pressure of adenosine deaminases acting on RNA. J Matern-fetal Neonatal Med 1-4.

Lorenzo, G., Martin-Folgar, R., Hevia, E., Boshra, H., Brun, A., 2010. Protection against lethal Rift Valley fever virus (RVFV) infection in transgenic IFNAR(-/-) mice induced by different DNA vaccination regimens. Vaccine 28, 2937-2944.

Luedke, A.J., Anakwenze, E.I., 1972. Bluetongue virus in goats. Am J Vet Res 33, 1739-1745.

Lymperopoulos, K., Noad, R., Tosi, S., Nethisinghe, S., Brierley, I., Roy, P., 2006. Specific binding of Bluetongue virus NS2 to different viral plus-strand RNAs. Virology 353, 17-26.

Lymperopoulos, K., Wirblich, C., Brierley, I., Roy, P., 2003. Sequence specificity in the interaction of Bluetongue virus non-structural protein 2 (NS2) with viral RNA. J Biol Chem 278, 31722-31730.

Maan, N.S., Maan, S., Belaganahalli, M.N., Ostlund, E.N., Johnson, D.J., Nomikou, K., Mertens, P.P., 2012. Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. PLoS One 7, e32601.

Maan, N.S., Maan, S., Nomikou, K., Guimera, M., Pullinger, G., Singh, K.P., Belaganahalli, M.N., Mertens, P.P., The genome sequence of bluetongue virus type 2 from India: evidence for reassortment between eastern and western topotype field strains. J Virol 86, 5967-5968.

Maan, S., Maan, N.S., Belaganahalli, M.N., Kumar, A., Batra, K., Rao, P.P., Hemadri, D., Reddy, Y.N., Putty, K., Krishnajyothi, Y., Reddy, G.H., Singh, K.P., Hegde, N.R., Nomikou, K., Sreenivasulu, D., Mertens, P.P., 2015. Genome Sequence of Bluetongue Virus Type 2 from India: Evidence for Reassortment between Outer Capsid Protein Genes. Genom Announ 3.

Maan, S., Maan, N.S., Nomikou, K., Veronesi, E., Bachanek-Bankowska, K., Belaganahalli, M.N., Attoui, H., Mertens, P.P., 2011. Complete genome characterisation of a novel 26th bluetongue virus serotype from kuwait. PLoS One 6, e26147.

Maan, S., Maan, N.S., Ross-smith, N., Batten, C.A., Shaw, A.E., Anthony, S.J., Samuel, A.R., Darpel, K.E., Veronesi, E., Oura, C.A., Singh, K.P., Nomikou, K., Potgieter, A.C., Attoui, H., van Rooij, E., van Rijn, P., De Clercq, K., Vandenbussche, F., Zientara, S., Breard, E., Sailleau, C., Beer, M., Hoffman, B., Mellor, P.S., Mertens, P.P., 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. Virology 377, 308-318.

Maan, S., Maan, N.S., Samuel, A.R., O'Hara, R., Meyer, A.J., Rao, S., Mertens, P.P., 2004. Completion of the sequence analysis and comparisons of genome segment 2 (encoding outer capsid protein VP2) from representative isolates of the 24 bluetongue virus serotypes. Vet Ital 40, 484-488.

Maan, S., Maan, N.S., Samuel, A.R., Rao, S., Attoui, H., Mertens, P.P., 2007a. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. J Gen Virol 88, 621-630.

Maan, S., Maan, N.S., van Rijn, P.A., van Gennip, R.G., Sanders, A., Wright, I.M., Batten, C., Hoffmann, B., Eschbaumer, M., Oura, C.A., Potgieter, A.C., Nomikou, K., Mertens, P.P., 2010. Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. PLoS One 5, e10323.

Maan, S., Rao, S., Maan, N.S., Anthony, S.J., Attoui, H., Samuel, A.R., Mertens, P.P., 2007b. Rapid cDNA synthesis and sequencing techniques for the genetic study of bluetongue and other dsRNA viruses. J Virol Methods 143, 132-139.

Maclachlan, N.J., 1994. The Pathogenesis and Immunology of Bluetongue Virus-Infection of Ruminants. Comparative Immunology Microbiology and Infectious Diseases 17, 197-206.

MacLachlan, N.J., 2004. Bluetongue: pathogenesis and duration of viraemia. Vet Ital 40, 462-467.

Maclachlan, N.J., 2010a. Global Implications of the Recent Emergence of Bluetongue Virus in Europe. Vet Clin N Am Food Anim Practice 26, 163-+.

Maclachlan, N.J., 2010b. Global implications of the recent emergence of bluetongue virus in Europe. Vet Clin North Am Food Anim Pract 26, 163-171, table of contents.

Maclachlan, N.J., 2011. Bluetongue: History, global epidemiology, and pathogenesis. Preventive Veterinary Medicine 102, 107-111.

MacLachlan, N.J., Crafford, J.E., Vernau, W., Gardner, I.A., Goddard, A., Guthrie, A.J., Venter, E.H., 2008. Experimental reproduction of severe bluetongue in sheep. Vet Pathol 45, 310-315.

Maclachlan, N.J., Drew, C.P., Darpel, K.E., Worwa, G., 2009. The Pathology and Pathogenesis of Bluetongue. Journal of Comparative Pathology 141, 1-16.

MacLachlan, N.J., Guthrie, A.J., 2010. Re-emergence of bluetongue, African horse sickness, and other Orbivirus diseases. Vet Res 41.

MacLachlan, N.J., Jagels, G., Rossitto, P.V., Moore, P.F., Heidner, H.W., 1990. The pathogenesis of experimental bluetongue virus infection of calves. Vet Pathol 27, 223-229.

Maclachlan, N.J., Schore, C.E., Osburn, B.I., 1984. Antiviral Responses of Bluetongue Virus-Inoculated Bovine Fetuses and Their Dams. Am J Vet Res 45, 1469-1473.

Maclachlan, N.J., Thompson, J., 1985. Bluetongue Virus-Induced Interferon in Cattle. Am Jl Vet Res 46, 1238-1241.

Mandl, C.W., Kroschewski, H., Allison, S.L., Kofler, R., Holzmann, H., Meixner, T., Heinz, F.X., 2001. Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo. J Virol 75, 5627-5637.

Martinez-Costas, J., Sutton, G., Ramadevi, N., Roy, P., 1998. Guanylyltransferase and RNA 5'-triphosphatase activities of the purified expressed VP4 protein of bluetongue virus. J Mol Biol 280, 859-866.

Matsuo, E., Roy, P., 2009. Bluetongue virus VP6 acts early in the replication cycle and can form the basis of chimeric virus formation. J Virol 83, 8842-8848.

Matsuo, E., Roy, P., 2011. Bluetongue Virus VP1 Polymerase Activity In Vitro: Template Dependency, Dinucleotide Priming and Cap Dependency. PLoS One 6, e27702.

Mayo, C.E., Mullens, B.A., Gerry, A.C., Barker, C.M., Mertens, P.P.C., Maan, S., Maan, N., Gardner, I.A., Guthrie, A.J., MacLachlan, N.J., 2012. The combination of abundance and infection rates of Culicoides sonorensis estimates risk of subsequent bluetongue virus infection of sentinel cattle on California dairy farms. Vet Parasitol 187, 295-301.

Mecham, J.O., Johnson, D.J., 2005. Persistence of bluetongue virus serotype 2 (BTV-2) in the southeast United States. Virus Res 113, 116-122.

Meiswinkel, R., Labuschagne, K., Baylis, M., Mellor, P.S., 2004. Multiple vectors and their differing ecologies: observations on two bluetongue and African horse sickness vector Culicoides species in South Africa. Vet Ital 40, 296-302.

Mellor, P., Oura, C., 2008. No room for complacency on bluetongue. Vet Rec 163, 697.

Mellor, P.S., 1990. The replication of bluetongue virus in Culicoides vectors. Curr Top Microbiol Immunol 162, 143-161.

Mellor, P.S., Baylis, M., Mertens, P.P.C 2009. Bluetongue. (Elsevier, Academic Press, London.).

Mellor, P.S., Hamblin, C., 2004. African horse sickness. Vet Res 35, 445-466.

Mellor, P.S., Osborne, R., Jennings, D.M., 1984. Isolation of bluetongue and related viruses from Culicoides spp. in the Sudan. J Hyg (Lond) 93, 621-628.

Mellor, P.S., Wittmann, E.J., 2002. Bluetongue virus in the Mediterranean Basin 1998-2001. Vet J 164, 20-37.

Mertens, P.P., Brown, F., Sangar, D.V., 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. Virology 135, 207-217.

Mertens, P.P., Burroughs, J.N., Walton, A., Wellby, M.P., Fu, H., O'Hara, R.S., Brookes, S.M., Mellor, P.S., 1996. Enhanced infectivity of modified bluetongue virus particles for two insect cell lines and for two Culicoides vector species. Virology 217, 582-593.

Mertens, P.P., Diprose, J., 2004. The bluetongue virus core: a nano-scale transcription machine. Virus Res 101, 29-43.

Mertens, P.P., Diprose, J., Maan, S., Singh, K.P., Attoui, H., Samuel, A.R., 2004. Bluetongue virus replication, molecular and structural biology. Vet Ital 40, 426-437.

Mertens, P.P., Pedley, S., Cowley, J., Burroughs, J.N., Corteyn, A.H., Jeggo, M.H., Jennings, D.M., Gorman, B.M., 1989. Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. Virology 170, 561-565.

Mertens, P.P., Sangar, D.V., 1985. Analysis of the terminal sequences of the genome segments of four orbiviruses. Prog Clin Biol Res 178, 371-387.

Milne, I., Bayer, M., Cardle, L., Shaw, P., Stephen, G., Wright, F., Marshall, D., 2010. Tablet--next generation sequence assembly visualization. Bioinformatics 26, 401-402.

Modrof, J., Lymperopoulos, K., Roy, P., 2005. Phosphorylation of bluetongue virus nonstructural protein 2 is essential for formation of viral inclusion bodies. J Virol 79, 10023-10031.

Monaco, F., Bonfini, B., Zaghini, M., Antonucci, D., Pini, A., Savini, G., 2004. Vaccination of cattle using monovalent modified-live vaccine against bluetongue virus serotype 2: innocuity, immunogenicity and effect on pregnancy. Vet Ital 40, 671-675.

Monastyrskaya, K., Booth, T., Nel, L., Roy, P., 1994. Mutation of either of two cysteine residues or deletion of the amino or carboxy terminus of nonstructural protein NS1 of bluetongue virus abrogates virus-specified tubule formation in insect cells. J Virol 68, 2169-2178.

Monastyrskaya, K., Gould, E.A., Roy, P., 1995. Characterization and modification of the carboxy-terminal sequences of bluetongue virus type 10 NS1 protein in relation to tubule formation and location of an antigenic epitope in the vicinity of the carboxy terminus of the protein. J Virol 69, 2831-2841.

Montville, R., Froissart, R., Remold, S.K., Tenaillon, O., Turner, P.E., 2005. Evolution of mutational robustness in an RNA virus. PLoS Biol 3, e381.

Moulin, V., Noordegraaf, C.V., Makoschey, B., van der Sluijs, M., Veronesi, E., Darpel, K., Mertens, P.P., de Smit, H., 2012. Clinical disease in sheep caused by bluetongue virus serotype 8, and prevention by an inactivated vaccine. Vaccine 30, 2228-2235.

Nason, E.L., Rothagel, R., Mukherjee, S.K., Kar, A.K., Forzan, M., Prasad, B.V., Roy, P., 2004. Interactions between the inner and outer capsids of bluetongue virus. J Virol 78, 8059-8067.

Neitz, W.O., 1948. Immunological studies on bluetongue in sheep. Onderstepoort J Vet Sci Anim Ind 23, 93-136.

Nomikou, K., Dovas, C.I., Maan, S., Anthony, S.J., Samuel, A.R., Papanastassopoulou, M., Maan, N.S., Mangana, O., Mertens, P.P., 2009. Evolution and phylogenetic analysis of full-length VP3 genes of Eastern Mediterranean bluetongue virus isolates. PLoS One 4, e6437.

Nomikou, K., Hughes, J., Wash, R., Kellam, P., Breard, E., Zientara, S., Palmarini, M., Biek, R., Mertens, P., 2015. Widespread Reassortment Shapes the Evolution and Epidemiology of Bluetongue Virus following European Invasion. PLoS Pathog 11, e1005056.

Nunes, S.F., Hamers, C., Ratinier, M., Shaw, A., Brunet, S., Hudelet, P., Palmarini, M., 2014. A synthetic biology approach for a vaccine platform against known and newly emerging serotypes of bluetongue virus. J Virol 88, 12222-12232.

O'Hara, R.S., Meyer, A.J., Burroughs, J.N., Pullen, L., Martin, L.A., Mertens, P.P., 1998. Development of a mouse model system, coding assignments and identification of the genome segments controlling virulence of African horse sickness virus serotypes 3 and 8. Arch Virol. Supplementum 14, 259-279.

Oberst, R.D., Stott, J.L., Blanchard-Channell, M., Osburn, B.I., 1987. Genetic reassortment of bluetongue virus serotype 11 strains in the bovine. Vet Microbiol 15, 11-18.

Olmsted, R.A., Baric, R.S., Sawyer, B.A., Johnston, R.E., 1984. Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. Science 225, 424-427.

Ortego, J., de la Poza, F., Marin-Lopez, A., 2014. Interferon alpha/beta receptor knockout mice as a model to study bluetongue virus infection. Virus Res 182, 35-42.

Osburn, B.I., 1994. The impact of bluetongue virus on reproduction. Comp Immunol Microbiol Infect Dis 17, 189-196.

Osburn, B.I., Johnson, R.T., Silverstein, A.M., Prendergast, R.A., Jochim, M.M., Levy, S.E., 1971a. Experimental viral-induced congenital encephalopathies. II. The pathogenesis of bluetongue vaccine virus infection in fetal lambs. Lab Invest 25, 206-210.

Osburn, B.I., Silverstein, A.M., Prendergast, R.A., Johnson, R.T., Parshall, C.J., Jr., 1971b. Experimental viral-induced congenital encephalopathies. I. Pathology of hydranencephaly and porencephaly caused by bluetongue vaccine virus. Lab Invest 25, 197-205.

Oura, C.A., Batten, C.A., Ivens, P.A., Balcha, M., Alhassan, A., Gizaw, D., Elharrak, M., Jallow, D.B., Sahle, M., Maan, N., Mertens, P.C., Maan, S., 2012. Equine encephalosis virus: evidence for circulation beyond southern Africa. Epidemiol Infect 140, 1982-1986.

Oura, C.A., Wood, J.L., Sanders, A.J., Bin-Tarif, A., Henstock, M., Edwards, L., Floyd, T., Simmons, H., Batten, C.A., 2009. Seroconversion, neutralising antibodies and protection in bluetongue serotype 8 vaccinated sheep. Vaccine 27, 7326-7330.

Owens, R.J., Limn, C., Roy, P., 2004. Role of an arbovirus nonstructural protein in cellular pathogenesis and virus release. J Virol 78, 6649-6656.

Pages, N., Breard, E., Urien, C., Talavera, S., Viarouge, C., Lorca-Oro, C., Jouneau, L., Charley, B., Zientara, S., Bensaid, A., Solanes, D., Pujols, J., Schwartz-Cornil, I., 2014. Culicoides midge bites modulate the host response and impact on bluetongue virus infection in sheep. PLoS One 9, e83683.

Parisien, J.P., Lau, J.F., Horvath, C.M., 2002. STAT2 acts as a host range determinant for species-specific paramyxovirus interferon antagonism and simian virus 5 replication. J Virol 76, 6435-6441.

Parrish, C.R., Holmes, E.C., Morens, D.M., Park, E.C., Burke, D.S., Calisher, C.H., Laughlin, C.A., Saif, L.J., Daszak, P., 2008. Cross-species virus transmission and the emergence of new epidemic diseases. Microbiology and molecular biology reviews : MMBR 72, 457-470.

Parsonson, I.M., 1990. Pathology and pathogenesis of bluetongue infections. Curr Top Microbiol Immunol 162, 119-141.

Pedley, S., Mohamed, M.E., Mertens, P.P., 1988. Analysis of genome segments from six different isolates of bluetongue virus using RNA-RNA hybridisation: a generalised coding assignment for bluetongue viruses. Virus Res 10, 381-390.

Perez-Cidoncha, M., Killip, M.J., Oliveros, J.C., Asensio, V.J., Fernandez, Y., Bengoechea, J.A., Randall, R.E., Ortin, J., 2014. An unbiased genetic screen reveals the polygenic nature of the influenza virus anti-interferon response. J Virol 88, 4632-4646.

Perrin, J.B., Ducrot, C., Vinard, J.L., Morignat, E., Gauffier, A., Calavas, D., Hendrikx, P., 2010. Using the National Cattle Register to estimate the excess mortality during an epidemic: application to an outbreak of Bluetongue serotype 8. Epidemics 2, 207-214.

Pini, A., 1976. Study on the pathogenesis of bluetongue: replication of the virus in the organs of infected sheep. Onderstepoort J Vet Res 43, 159-164.

Purdy, M.A., Ghiasi, H., Rao, C.D., Roy, P., 1985. Complete sequence of bluetongue virus L2 RNA that codes for the antigen recognized by neutralizing antibodies. J Virol 55, 826-830.

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P., Baylis, M., 2005. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol 3, 171-181.

Purse, B.V., Tatem, A.J., Caracappa, S., Rogers, D.J., Mellor, P.S., Baylis, M., Torina, A., 2004. Modelling the distributions of Culicoides bluetongue virus vectors in Sicily in relation to satellite-derived climate variables. Med Vet Entomol 18, 90-101.

Ramadevi, N., Burroughs, N.J., Mertens, P.P., Jones, I.M., Roy, P., 1998. Capping and methylation of mRNA by purified recombinant VP4 protein of bluetongue virus. Proc Natl Acad Sci U S A 95, 13537-13542.

Ramadevi, N., Roy, P., 1998. Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity. J Gen Virol 79 (Pt 10), 2475-2480.

Ramig, R.F., Garrison, C., Chen, D., Bell-Robinson, D., 1989. Analysis of reassortment and superinfection during mixed infection of Vero cells with bluetongue virus serotypes 10 and 17. J Gen Virol 70 (Pt 10), 2595-2603.

Randall, R.E., Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol 89, 1-47.

Rasmussen, L.D., Savini, G., Lorusso, A., Bellacicco, A., Palmarini, M., Caporale, M., Rasmussen, T.B., Belsham, G.J., Botner, A., 2013. Transplacental transmission of field and rescued strains of BTV-2 and BTV-8 in experimentally infected sheep. Vet Res 44, 75.

Ratinier, M., Caporale, M., Golder, M., Franzoni, G., Allan, K., Nunes, S.F., Armezzani, A., Bayoumy, A., Rixon, F., Shaw, A., Palmarini, M., 2011. Identification and characterization of a novel non-structural protein of bluetongue virus. PLoS Pathog 7, e1002477.

Richardson, C., Taylor, W.P., Terlecki, S., Gibbs, E.P., 1985. Observations on transplacental infection with bluetongue virus in sheep. Am J Vet Res 46, 1912-1922.

Rima, B.K., Gatherer, D., Young, D.F., Norsted, H., Randall, R.E., Davison, A.J., 2014. Stability of the parainfluenza virus 5 genome revealed by deep sequencing of strains isolated from different hosts and following passage in cell culture. J Virol 88, 3826-3836.

Rojas, J.M., Rodriguez-Calvo, T., Pena, L., Sevilla, N., 2011. T cell responses to bluetongue virus are directed against multiple and identical CD4+ and CD8+ T cell epitopes from the VP7 core protein in mouse and sheep. Vaccine 29, 6848-6857.

Rose, T.L., Marques da Silva, M.F., Gomez, M.M., Resque, H.R., Ichihara, M.Y., Volotao Ede, M., Leite, J.P., 2013. Evidence of vaccine-related reassortment of rotavirus, Brazil, 2008-2010. Emerg Infect Dis 19, 1843-1846.

Roy, P., 2005. Bluetongue virus proteins and particles and their role in virus entry, assembly, and release. Adv Virus Res 64, 69-123.

Roy, P., 2008a. Bluetongue virus: dissection of the polymerase complex. J Gen Virol 89, 1789-1804.

Roy, P., 2008b. Functional mapping of bluetongue virus proteins and their interactions with host proteins during virus replication. Cell Biochem Biophys 50, 143-157.

Roy, P., Adachi, A., Urakawa, T., Booth, T.F., Thomas, C.P., 1990. Identification of bluetongue virus VP6 protein as a nucleic acid-binding protein and the localization of VP6 in virus-infected vertebrate cells. J Virol 64, 1-8.

Roy, P., Boyce, M., Noad, R., 2009. Prospects for improved bluetongue vaccines. Nat Rev Microbiol 7, 120-128.

Ruder, M.G., Allison, A.B., Stallknecht, D.E., Mead, D.G., McGraw, S.M., Carter, D.L., Kubiski, S.V., Batten, C.A., Klement, E., Howerth, E.W., 2012. Susceptibility of white-tailed deer (Odocoileus virginianus) to experimental infection with epizootic hemorrhagic disease virus serotype 7. J Wildl Dis 48, 676-685.

Ruscanu, S., Pascale, F., Bourge, M., Hemati, B., Elhmouzi-Younes, J., Urien, C., Bonneau, M., Takamatsu, H., Hope, J., Mertens, P., Meyer, G., Stewart, M., Roy, P., Meurs, E.F., Dabo, S., Zientara, S., Breard, E., Sailleau, C., Chauveau, E., Vitour, D., Charley, B.,

Schwartz-Cornil, I., The double-stranded RNA bluetongue virus induces type I interferon in plasmacytoid dendritic cells via a MYD88-dependent TLR7/8-independent signaling pathway. J Virol 86, 5817-5828.

Ruscanu, S., Pascale, F., Bourge, M., Hemati, B., Elhmouzi-Younes, J., Urien, C., Bonneau, M., Takamatsu, H., Hope, J., Mertens, P., Meyer, G., Stewart, M., Roy, P., Meurs, E.F., Dabo, S., Zientara, S., Breard, E., Sailleau, C., Chauveau, E., Vitour, D., Charley, B., Schwartz-Cornil, I., 2012. The double-stranded RNA bluetongue virus induces type I interferon in plasmacytoid dendritic cells via a MYD88-dependent TLR7/8-independent signaling pathway. J Virol 86, 5817-5828.

Russell, H., O'Toole, D.T., Bardsley, K., Davis, W.C., Ellis, J.A., 1996. Comparative effects of bluetongue virus infection of ovine and bovine endothelial cells. Vet Pathol 33, 319-331.

Sabin, A.B., 1959. Reoviruses. A new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. Science 130, 1387-1389.

Saegerman, C., Berkvens, D., Mellor, P.S., 2008. Bluetongue epidemiology in the European Union. Emerg Infect Dis 14, 539-544.

Samal, S.K., el-Hussein, A., Holbrook, F.R., Beaty, B.J., Ramig, R.F., 1987a. Mixed infection of Culicoides variipennis with bluetongue virus serotypes 10 and 17: evidence for high frequency reassortment in the vector. J Gen Virol 68 (Pt 9), 2319-2329.

Samal, S.K., Livingston, C.W., Jr., McConnell, S., Ramig, R.F., 1987b. Analysis of mixed infection of sheep with bluetongue virus serotypes 10 and 17: evidence for genetic reassortment in the vertebrate host. J Virol 61, 1086-1091.

Sanchez-Cordon, P.J., Pleguezuelos, F.J., Perez de Diego, A.C., Gomez-Villamandos, J.C., Sanchez-Vizcaino, J.M., Ceron, J.J., Tecles, F., Garfia, B., Pedrera, M., 2013. Comparative study of clinical courses, gross lesions, acute phase response and coagulation disorders in sheep inoculated with bluetongue virus serotype 1 and 8. Vet Microbiol 166, 184-194.

Santman-Berends, I.M., Hage, J.J., van Rijn, P.A., Stegeman, J.A., van Schaik, G., 2010a. Bluetongue virus serotype 8 (BTV-8) infection reduces fertility of Dutch dairy cattle and is vertically transmitted to offspring. Theriogenology 74, 1377-1384.

Santman-Berends, I.M., van Wuijckhuise, L., Vellema, P., van Rijn, P.A., 2010b. Vertical transmission of bluetongue virus serotype 8 virus in Dutch dairy herds in 2007. Vet Microbiol 141, 31-35.

Sauder, C.J., Vandenburgh, K.M., Iskow, R.C., Malik, T., Carbone, K.M., Rubin, S.A., 2006. Changes in mumps virus neurovirulence phenotype associated with quasispecies heterogeneity. Virology 350, 48-57.

Savini, G., Afonso, A., Mellor, P., Aradaib, I., Yadin, H., Sanaa, M., Wilson, W., Monaco, F., Domingo, M., 2011. Epizootic heamorragic disease. Res Vet Sci 91, 1-17.

Savini, G., Cannas, A., Casaccia, C., Di Gialleonardo, L., Leone, A., Patta, C., Nicolussi, P., 2010. Risk factors associated with the occurrence of undesired effects in sheep and goats after field vaccination with modified-live vaccine against bluetongue virus serotypes 2, 4 and 16. Vet Microbiol 146, 44-50.

Savini, G., Goffredo, M., Monaco, F., Di Gennaro, A., Cafiero, M.A., Baldi, L., de Santis, P., Meiswinkel, R., Caporale, V., 2005. Bluetongue virus isolations from midges belonging to the Obsoletus complex (Culicoides, Diptera: Ceratopogonidae) in Italy. Vet Rec 157, 133-139.

Savini, G., Goffredo, M., Monaco, F., Di Gennaro, A., de Santis, P., Meiswinkel, R., Caporale, V., 2004. The isolation of bluetongue virus from field populations of the Obsoletus Complex in central Italy. Vet Ital 40, 286-291.

Savini, G., MacLachlan, N.J., Sanchez-Vizcaino, J.M., Zientara, S., 2008. Vaccines against bluetongue in Europe. Comp Immunol Microbiol Infect Dis 31, 101-120.

Savini, G., Ronchi, G.F., Leone, A., Ciarelli, A., Migliaccio, P., Franchi, P., Mercante, M.T., Pini, A., 2007. An inactivated vaccine for the control of bluetongue virus serotype 16 infection in sheep in Italy. Vet Microbiol 124, 140-146.

Schnettler, E., Ratinier, M., Watson, M., Shaw, A.E., McFarlane, M., Varela, M., Elliott, R.M., Palmarini, M., Kohl, A., 2013. RNA interference targets arbovirus replication in Culicoides cells. J Virol 87, 2441-2454.

Schultz, G., Delay, P.D., 1955. Losses in newborn lambs associated with bluetongue vaccination of pregnancy ewes. J Am Vet Med Assoc 127, 224-226.

Schultz, K.T., Grieder, F.B., 1987. Immunobiology of bluetongue virus. Vet Immunol Immunopathol 15, 115-127.

Schwartz-Cornil, I., Mertens, P.P.C., Contreras, V., Hemati, B., Pascale, F., Breard, E., Mellor, P.S., MacLachlan, N.J., Zientara, S., 2008. Bluetongue virus: virology, pathogenesis and immunity. Vet Res 39.

Sealfon, R.S., Lin, M.F., Jungreis, I., Wolf, M.Y., Kellis, M., Sabeti, P.C., 2015. FRESCo: finding regions of excess synonymous constraint in diverse viruses. Gen biolog 16, 38.

Sellers, R.F., 1975. Bluetongue in Cyprus. Aust Vet J 51, 198-203.

Sellers, R.F., 1984. Bluetongue in Africa, the Mediterranean Region and near-East - Disease, Virus and Vectors. Prev Vet Med 2, 371-378.

Shaw, A.E., Monaghan, P., Alpar, H.O., Anthony, S., Darpel, K.E., Batten, C.A., Guercio, A., Alimena, G., Vitale, M., Bankowska, K., Carpenter, S., Jones, H., Oura, C.A., King, D.P., Elliott, H., Mellor, P.S., Mertens, P.P., 2007. Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. J Virol Methods 145, 115-126.

Shaw, A.E., Ratinier, M., Nunes, S.F., Nomikou, K., Caporale, M., Golder, M., Allan, K., Hamers, C., Hudelet, P., Zientara, S., Breard, E., Mertens, P., Palmarini, M., 2013. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. J Virol 87, 543-557.

Silva, S.P., Dilcher, M., Weber, F., Hufert, F.T., Weidmann, M., Cardoso, J.F., Carvalho, V.L., Chiang, J.O., Martins, L.C., Lima, C.P., Da Silva, D.E., Vianez-Junior, J.L., Popov, V.L., Travassos da Rosa, A.P., Tesh, R.B., Vasconcelos, P.F., Nunes, M.R., 2014. Genetic and biological characterization of selected Changuinola viruses (Reoviridae, Orbivirus) from Brazil. J Gen Virol 95, 2251-2259.

Spiropoulou, C.F., Albarino, C.G., Ksiazek, T.G., Rollin, P.E., 2007. Andes and Prospect Hill hantaviruses differ in early induction of interferon although both can downregulate interferon signaling. J Virol 81, 2769-2776.

Spreull, J., 1905. Malarial catarrhal fever (bluetongue) of sheep in South Africa. J Comp Path Ther 18, 321-339.

Stauber, N., Martinez-Costas, J., Sutton, G., Monastyrskaya, K., Roy, P., 1997. Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function and a helicase activity that catalyze the unwinding of double-stranded RNA substrates. J Virol 71, 7220-7226.

Stewart, M., Dovas, C.I., Chatzinasiou, E., Athmaram, T.N., Papanastassopoulou, M., Papadopoulos, O., Roy, P., 2012. Protective efficacy of Bluetongue virus-like and subvirus-like particles in sheep: presence of the serotype-specific VP2, independent of its geographic lineage, is essential for protection. Vaccine 30, 2131-2139.

Stewart, M., Dubois, E., Sailleau, C., Breard, E., Viarouge, C., Desprat, A., Thiery, R., Zientara, S., Roy, P., 2013. Bluetongue virus serotype 8 virus-like particles protect sheep

against virulent virus infection as a single or multi-serotype cocktail immunogen. Vaccine 31, 553-558.

Stewart, M., Hardy, A., Barry, G., Pinto, R.M., M., C., Melzi, E., Hughes, J., Taggart, A., Janowicz, A., Varela, M., Ratinier, M., Palmarini, M., 2015. Characterisation of a second open reading frame in genome segment 10 of bluetongue virus. J Gen Virol.

Stewart, M.E., Roy, P., 2015. Structure-based identification of functional residues in the nucleoside-2'-O-methylase domain of Bluetongue virus VP4 capping enzyme. FEBS open bio 5, 138-146.

Stott, J.L., Oberst, R.D., Channell, M.B., Osburn, B.I., 1987. Genome segment reassortment between two serotypes of bluetongue virus in a natural host. J Virol 61, 2670-2674.

Stuart, D.I., Grimes, J.M., 2006. Structural studies on orbivirus proteins and particles. Curr Top Microbiol Immunol 309, 221-244.

Sugiyama, K., Bishop, D.H., Roy, P., 1982. Analysis of the genomes of bluetongue viruses recovered from different states of the United States and at different times. Am J Epidemiol 115, 332-347.

Sung, P.Y., Roy, P., 2014. Sequential packaging of RNA genomic segments during the assembly of Bluetongue virus. Nucleic Acids Res 42, 13824-13838.

Sutton, G., Grimes, J.M., Stuart, D.I., Roy, P., 2007. Bluetongue virus VP4 is an RNA-capping assembly line. Nat Struct Mol Biol 14, 449-451.

Szmaragd, C., Wilson, A., Carpenter, S., Mertens, P.P., Mellor, P.S., Gubbins, S., 2007. Mortality and case fatality during the recurrence of BTV-8 in northern Europe in 2007. Vet Rec 161, 571-572.

Szmaragd, C., Wilson, A.J., Carpenter, S., Wood, J.L., Mellor, P.S., Gubbins, S., 2010. The spread of bluetongue virus serotype 8 in Great Britain and its control by vaccination. PLoS One 5, e9353.

Tabachnick, W.J., 1991. Genetic control of oral susceptibility to infection of Culicoides variipennis with bluetongue virus. Am J Trop Med Hyg 45, 666-671.

Tabachnick, W.J., 2004. Culicoides and the global epidemiology of bluetongue virus infection. Vet Ital 40, 144-150.

Takamatsu, H., Mellor, P.S., Mertens, P.P., Kirkham, P.A., Burroughs, J.N., Parkhouse, R.M., 2003. A possible overwintering mechanism for bluetongue virus in the absence of the insect vector. J Gen Virol 84, 227-235.

Tan, B.H., Nason, E., Staeuber, N., Jiang, W., Monastryrskaya, K., Roy, P., 2001. RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to Culicoides cells. J Virol 75, 3937-3947.

Tanaka, S., Mikhailov, M., Roy, P., 1995. Synthesis of bluetongue virus chimeric VP3 molecules and their interactions with VP7 protein to assemble into virus core-like particles. Virology 214, 593-601.

Taraporewala, Z.F., Chen, D., Patton, J.T., 2001. Multimers of the bluetongue virus nonstructural protein, NS2, possess nucleotidyl phosphatase activity: similarities between NS2 and rotavirus NSP2. Virology 280, 221-231.

Theiler, A., 1908. The inoculation of sheep against bluetongue and the results in practice. Vet J 64, 600-607.

Thiry, E., Saegerman, C., Guyot, H., Kirten, P., Losson, B., Rollin, F., Bodmer, M., Czaplicki, G., Toussaint, J.F., De Clercq, K., Dochy, J.M., Dufey, J., Gilleman, J.L., Messeman, K., 2006. Bluetongue in northern Europe. Vet Rec 159, 327.

Thomas, C.P., Booth, T.F., Roy, P., 1990. Synthesis of bluetongue virus-encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. J Gen Virol 71 (Pt 9), 2073-2083.

Thomas, F.C., Trainer, D.O., 1970. Bluetongue virus in white-tailed deer. Am J Vet Res 31, 271-278.

Tsugawa, T., Tatsumi, M., Tsutsumi, H., 2014. Virulence-associated genome mutations of murine rotavirus identified by alternating serial passages in mice and cell cultures. J Virol 88, 5543-5558.

Urakawa, T., Ritter, D.G., Roy, P., 1989. Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. Nucleic Acids Res 17, 7395-7401.

Urbano, P., Urbano, F.G., 1994. The Reoviridae family. Comp Immunol Microbiol Infect Dis 17, 151-161.

van der Sluijs, M.T., Schroer-Joosten, D.P., Fid-Fourkour, A., Vrijenhoek, M.P., Debyser, I., Moulin, V., Moormann, R.J., de Smit, A.J., 2013. Transplacental transmission of Bluetongue virus serotype 1 and serotype 8 in sheep: virological and pathological findings. PLoS One 8, e81429.

van der Walt, N.T., 1980. A haemagglutination and haemagglutination inhibition test for bluetongue virus. Onderstepoort J Vet Res 47, 113-117.

Van Dijk, A.A., Huismans, H., 1980. The in vitro activation and further characterization of the bluetongue virus-associated transcriptase. Virology 104, 347-356.

Van Dijk, A.A., Huismans, H., 1982. The effect of temperature on the in vitro transcriptase reaction of bluetongue virus, epizootic haemorrhagic disease virus and African horsesickness virus. Onderstepoort J Vet Res 49, 227-232.

Van Dijk, A.A., Huismans, H., 1988. In vitro transcription and translation of bluetongue virus mRNA. J Gen Virol 69 (Pt 3), 573-581.

van Gennip, R.G., van de Water, S.G., van Rijn, P.A., 2014. Bluetongue virus nonstructural protein NS3/NS3a is not essential for virus replication. PLoS One 9, e85788.

van Gennip, R.G., Veldman, D., van de Water, S.G., van Rijn, P.A., 2010. Genetic modification of Bluetongue virus by uptake of "synthetic" genome segments. Virol J 7, 261.

van Rijn, P.A., Geurts, Y., van der Spek, A.N., Veldman, D., van Gennip, R.G., 2012. Bluetongue virus serotype 6 in Europe in 2008-Emergence and disappearance of an unexpected non-virulent BTV. Vet Microbiol 158, 23-32.

van Schaik, G., Berends, I.M., van Langen, H., Elbers, A.R., Vellema, P., 2008. Seroprevalence of bluetongue serotype 8 in cattle in the Netherlands in spring 2007, and its consequences. Vet Rec 163, 441-444.

Vandenbussche, F., Vanbinst, T., Verheyden, B., Van Dessel, W., Demeestere, L., Houdart, P., Bertels, G., Praet, N., Berkvens, D., Mintiens, K., Goris, N., De Clercq, K., 2008. Evaluation of antibody-ELISA and real-time RT-PCR for the diagnosis and profiling of bluetongue virus serotype 8 during the epidemic in Belgium in 2006. Vet Microbiol 129, 15-27.

Varela, M., Schnettler, E., Caporale, M., Murgia, C., Barry, G., McFarlane, M., McGregor, E., Piras, I.M., Shaw, A., Lamm, C., Janowicz, A., Beer, M., Glass, M., Herder, V., Hahn, K., Baumgartner, W., Kohl, A., Palmarini, M., 2013. Schmallenberg virus pathogenesis, tropism and interaction with the innate immune system of the host. PLoS Pathog 9, e1003133.

Veronesi, E., Darpel, K.E., Hamblin, C., Carpenter, S., Takamatsu, H.H., Anthony, S.J., Elliott, H., Mertens, P.P., Mellor, P.S., 2010. Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4. Vaccine 28, 1397-1403.

Verwoerd, D.W., Huismans, H., 1972. Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. Onderstepoort J Vet Res 39, 185-191.

Vignuzzi, M., Stone, J.K., Arnold, J.J., Cameron, C.E., Andino, R., 2006. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. Nature 439, 344-348.

Viljoen, G.J., Huismans, H., 1989. The characterization of equine encephalosis virus and the development of genomic probes. J Gen Virol 70 (Pt 8), 2007-2015.

Vitour, D., Doceul, V., Ruscanu, S., Chauveau, E., Schwartz-Cornil, I., Zientara, S., 2014. Induction and control of the type I interferon pathway by Bluetongue virus. Virus Res 182, 59-70.

von Teichman, B.F., Dungu, B., Smit, T.K., 2010. In vivo cross-protection to African horse sickness Serotypes 5 and 9 after vaccination with Serotypes 8 and 6. Vaccine 28, 6505-6517.

Wackerlin, R., Eschbaumer, M., Konig, P., Hoffmann, B., Beer, M., 2010. Evaluation of humoral response and protective efficacy of three inactivated vaccines against bluetongue virus serotype 8 one year after vaccination of sheep and cattle. Vaccine 28, 4348-4355.

Waldvogel, A.S., Anderson, C.A., Higgins, R.J., Osburn, B.I., 1987. Neurovirulence of the UC-2 and UC-8 strains of bluetongue virus serotype 11 in newborn mice. Vet Pathol 24, 404-410.

Waldvogel, A.S., Anderson, G.A., Phillips, D.L., Osburn, B.I., 1992. Infection of bovine fetuses at 120 days' gestation with virulent and avirulent strains of bluetongue virus serotype 11. Comp Immunol Microbiol Infect Dis 15, 53-63.

Waldvogel, A.S., Stott, J.L., Squire, K.R., Osburn, B.I., 1986. Strain-dependent virulence characteristics of bluetongue virus serotype 11. J Gen Virol 67 (Pt 4), 765-769.

Webby, R., Hoffmann, E., Webster, R., 2004. Molecular constraints to interspecies transmission of viral pathogens. Nat Med 10, S77-81.

Wehrfritz, J.M., Boyce, M., Mirza, S., Roy, P., 2007. Reconstitution of bluetongue virus polymerase activity from isolated domains based on a three-dimensional structural model. Biopolymers 86, 83-94.

Westcott, D., Mildenberg, Z., Bellaiche, M., McGowan, S.L., Grierson, S.S., Choudhury, B., Steinbach, F., 2013. Evidence for the circulation of equine encephalosis virus in Israel since 2001. PLoS One 8, e70532.

Whetter, L.E., Maclachlan, N.J., Gebhard, D.H., Heidner, H.W., Moore, P.F., 1989. Bluetongue Virus-Infection of Bovine Monocytes. J Gen Virol 70, 1663-1676.

Williamson, S., Woodger, N., Darpel, K., 2008. Differential diagnosis of bluetongue in cattle and sheep. In Practice 30, 242-251.

Wilson, A., Darpel, K., Mellor, P.S., 2008. Where does bluetongue virus sleep in the winter? PLoS Biol 6, e210.

Wilson, A., Mellor, P.S., Szmaragd, C., Mertens, P.P., 2009. Adaptive strategies of African horse sickness virus to facilitate vector transmission. Vet Res 40, 16.

Wilson, A.J., Mellor, P.S., 2009. Bluetongue in Europe: past, present and future. Philos Trans R Soc Lond B Biol Sci 364, 2669-2681.

Wirblich, C., Bhattacharya, B., Roy, P., 2006. Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. J Virol 80, 460-473.

Worwa, G., Hilbe, M., Ehrensperger, F., Chaignat, V., Hofmann, M.A., Griot, C., Maclachlan, N.J., Thuer, B., 2009. Experimental transplacental infection of sheep with bluetongue virus serotype 8. Vet Rec 164, 499-500.

Worwa, G., Thur, B., Griot, C., Hofinann, M., Maclachlan, J.N., Chaignat, V., 2008. Clinical signs observed in Swiss sheep after experimental bluetongue virus serotype 8 infection. Schweizer Archiv Fur Tierheilkunde 150, 491-498.

Xu, G., Wilson, W., Mecham, J., Murphy, K., Zhou, E.M., Tabachnick, W., 1997. VP7: an attachment protein of bluetongue virus for cellular receptors in Culicoides variipennis. J Gen Virol 78 (Pt 7), 1617-1623.

Zahn, R.C., Schelp, I., Utermohlen, O., von Laer, D., 2007. A-to-G hypermutation in the genome of lymphocytic choriomeningitis virus. J Virol 81, 457-464.

Zhang, X., Boyce, M., Bhattacharya, B., Schein, S., Roy, P., Zhou, Z.H., 2010. Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5 resembles enveloped virus fusion proteins. Proc Natl Acad Sci U S A 107, 6292-6297.

Zust, R., Dong, H., Li, X.F., Chang, D.C., Zhang, B., Balakrishnan, T., Toh, Y.X., Jiang, T., Li, S.H., Deng, Y.Q., Ellis, B.R., Ellis, E.M., Poidinger, M., Zolezzi, F., Qin, C.F., Shi, P.Y., Fink, K., 2013. Rational design of a live attenuated dengue vaccine: 2'-o-methyltransferase mutants are highly attenuated and immunogenic in mice and macaques. PLoS Pathog 9, e1003521.

Zwart, L., Potgieter, C.A., Clift, S.J., van Staden, V., 2015. Characterising Non-Structural Protein NS4 of African Horse Sickness Virus. PLoS One 10, e0124281.