Bluetongue virus genetic and phenotypic diversity: towards identifying the molecular determinants that influence virulence and transmission potential

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Abstract

Bluetongue virus (BTV) is the prototype member of the *Orbivirus* genus in the family *Reoviridae* and is the aetiological agent of the arthropod transmitted disease bluetongue (BT) that affects both ruminant and camelid species. The disease is of significant global importance due to its economic impact and effect on animal welfare. Bluetongue virus, a dsRNA virus, evolves through a process of quasispecies evolution that is driven by genetic drift and shift as well as intragenic recombination. Quasispecies evolution coupled with founder effect and evolutionary selective pressures has over time led to the establishment of genetically distinct strains of the virus in different epidemiological systems throughout the world.

Bluetongue virus field strains may differ substantially from each other with regards to their phenotypic properties (i.e. virulence and/or transmission potential). The intrinsic molecular determinants that influence the phenotype of BTV have not yet clearly been characterized. It is currently unclear what contribution each of the viral genome segments have in determining the phenotypic properties of the virus and it is also unknown how genetic variability in the individual viral genes and their functional domains relate to differences in phenotype.

In order to understand how genetic variation in particular viral genes could potentially influence the phenotypic properties of the virus; a closer understanding of the BTV virion, its

encoded proteins and the evolutionary mechanisms that shape the diversity of the virus is required. This review provides a synopsis of these issues and highlights some of the studies that have been conducted on BTV and the closely related African horse sickness virus (AHSV) that have contributed to ongoing attempts to identify the molecular determinants that influence the virus' phenotype. Different strategies that can be used to generate BTV mutants *in vitro* and methods through which the causality between particular genetic modifications and changes in phenotype may be determined are also described. Finally examples are highlighted where a clear understanding of the molecular determinants that influence the phenotype of the virus may have contributed to risk assessment and mitigation strategies during recent outbreaks of BT in Europe.

Keywords

Bluetongue virus, bluetongue, genetic drift, genetic shift, intragenic recombination, phenotype, virulence, transmission potential, reverse genetics, modified-live virus vaccine strain.

General introduction

Bluetongue virus (BTV) is the type species of the genus *Orbivirus* in the family *Reoviridae* (Borden *et al.*, 1971) and causes an infectious, non-contagious, arthropod transmitted disease of ruminants and camelids called bluetongue (BT) (Spreull, 1905). Twenty six distinct serotypes of the virus have been identified to date (Chaignat *et al.*, 2009; Howell *et al.*, 1970; Maan *et al.*, 2011). Bluetongue virus is transmitted primarily by biting midges that belong to the *Culicoides* genus (Diptera: Ceratopogonidae) (Du Toit, 1944). The distribution of the virus closely matches the distribution of vector-competent midge species and climatic conditions that support a large

population of these insects. The virus therefore occurs more commonly in tropical and sub-tropical regions of the world between the latitudes of 40-50 °N and 35 °S and during times of the year that are optimal for vector activity (Gibbs and Greiner, 1994; Mellor *et al.*, 2000).

Bluetongue virus infection in ruminants is characterized by damage to endothelial cells lining small caliber blood vessels resulting in vascular thrombosis, ischaemic necrosis, haemorrhage and vascular leakage (Erasmus, 1975a; Mahrt and Osburn, 1986). Clinical signs of BT in sheep may include any combination of fever, depression, anorexia, nasal discharge, facial and pulmonary oedema, coronitis, erosions of the mucosa of the buccal cavity, occasional cyanosis of the tongue and muscle degeneration (Maclachlan *et al.*, 2008; Spreull, 1905). Bluetongue virus causes severe disease in certain breeds of sheep (especially European fine wool and mutton breeds) (Spreull, 1905) and certain species of deer (Vosdingh *et al.*, 1968), whereas cattle and goats are usually sub-clinically affected (Anderson *et al.*, 1985; Luedke and Anakwenze, 1972). The mortality of the disease amongst susceptible sheep can range from 2-30% during mild outbreaks (Verwoerd et al., 2004), but can occasionally be as high as 70% (Gambles, 1949).

In endemic regions local ruminant breeds demonstrate resistance against the disease. Overt clinical signs are therefore usually only observed when susceptible breeds are imported into BT endemic regions or when the virus is introduced into immunologically naïve flocks in regions where it is not normally encountered (Gibbs and Greiner, 1994). The clinical presentation of BT also varies even amongst susceptible sheep, ranging from sub-clinical to acute disease that can lead to the death of infected animals. This variation in severity is influenced both by intrinsic

differences in virulence between infecting field strains as well as extrinsic host, vector and environmental factors (breed, age, nutritional status, level of immunity, inoculum titre, temperature, UV radiation (Erasmus, 1975a).

Genetic and phenotypic diversity

Bluetongue virus circulates in different regions of the world in distinct ecological zones or that are defined by the occurrence of particular *Culicoides* populations (Tabachnick, 2004). The localized circulation of the virus in these host-vector defined 'episystems' has over time led to the evolution of distinct geographical strains or topotypes of the virus. These viruses are broadly divided into western and eastern lineages and further regional subtypes based on phylogenetic analysis of sequence data of the majority of viral genome segments (Gould, 1987; Gould and Pritchard, 1990; Maan *et al.*, 2010). The virus also demonstrates substantial genetic variation even amongst strains that circulate in the same geographical region. For example it has been demonstrated that the nucleotide sequence of the NS3/A encoding gene of BTV field isolates collected from pools of *C. sonorenis* from a single dairy in California in the United States, varied over a three month period from 97.54 to 100% nucleotide sequence identity (Bonneau *et al.*, 2002).

Genetically heterogeneous BTV field strains may vary substantially from each other with regards to their phenotypic properties (i.e. virulence and transmission potential). For example, differences have been noted to exist in the virulence of BTV-4 from South Africa and BTV-4 that circulates in the Americas (South America, Central America, and Caribbean). Bluetongue virus serotype 4 from South Africa is highly virulent when inoculated experimentally into Merino

sheep (Maclachlan *et al*., 2008), whereas BTV-4 that circulates in the Americas appears to be less virulent and is rarely associated with clinical disease in the field (Gibbs and Greiner, 1994). In addition it has been documented that certain strains of BTV in South Africa are more often isolated from *Culicoides* and are therefore thought to have a high transmission potential, whereas others are more often isolated from acutely affected sheep and appear to have a high pathogenic index (Anonymous, 1985; Nevill *et al*., 1992). Differences have also been reported to exist in virulence between Australian and South African strains of BTV, with Australian strains generally being considered to be less virulent than strains from South Africa (Kirkland, 2004).

Modified-live virus vaccine strains have been attenuated from wild type strains in South Africa and the United States by sequentially passaging field isolates in embryonated chicken eggs and cell culture. The sequential passage of BTV in these atypical host systems results in the attenuation of virulent field strains, presumably by selecting for quasispecies variants that have a reduced ability to infect and replicate in the ruminant host (Erasmus, 1975b; Walton, 1992). Differences in BTV phenotype are also emphasized when comparing modified-live virus vaccine strains (MLV) to wild type strains. Apart from a decrease in virulence, MLV strains differ from wild type strains in that they demonstrate novel biological properties. These include an increased tendency to cross the ruminant placenta when used in pregnant females resulting in infection of the foetus (Flanagan and Johnson, 1995; Schultz and Delay, 1955) as well as a possible increased tendency to be secreted in the semen of older rams and bulls (Kirkland and Hawkes, 2004). Modified-live virus vaccine strains may also possibly have an increased ability to infect and replicate in *Culicoides*. For example, studies on the closely related AHSV indicated that when an AHSV-7 MLV strain and corresponding field isolates of the same serotype were

evaluated in an oral susceptibility study in *C. imicola* and *C. bolitinos* in South Africa, recovery rates and viral titres of the vaccine strain were higher after the extrinsic incubation period had been completed than for wild type strains (Venter and Paweska, 2007)..

Structural and molecular biology of BTV

Bluetongue virus has a segmented genome that consists of 10 linear double stranded RNA segments (dsRNA) (Verwoerd *et al.*, 1970). The 10 viral genome segments encode seven structural (VP1-VP7) and five non-structural proteins (NS1, NS2, NS3, NS3A, NS4) (Ratinier *et al.*, 2011; van Dijk and Huismans, 1988). Each of the genome segments except segments 9 and 10 codes for a single protein. Segment 9 codes for both VP6 and non-structural protein 4 (NS4), with the latter being translated from an overlapping reading frame (Firth, 2008; Ratinier *et al.*, 2011). Similarly segment 10 codes for two proteins, with translation of NS3/A, a truncated version of full length NS3 lacking the N-terminal 13 amino acid residues, being initiated from an internal in-frame methionine codon (van Dijk and Huismans, 1988).

Viral proteins VP1 to VP7 constitute the structural components of the mature BTV virion, whereas the non-structural proteins (NS1-NS4) are only found in BTV infected cells (Ratinier *et al.*, 2011; van Dijk and Huismans, 1988). The mature BTV virion is non-enveloped, spherical in appearance and can be divided into an outer diffuse protein layer, a transcriptionally active core and an icosahedral sub-core. The sub-core is composed of 60 dimers of VP3 (coded for by segment 3) that are arranged with icosahedral symmetry. The sub-core is covered by 260 trimers of VP7 (coded for by segment 7) that curve around the surface of the sub-core (Grimes *et al.*, 1998; Hewat *et al.*, 1992; Huismans *et al.*, 1987; Verwoerd *et al.*, 1972). Epitopes of VP7

are exposed through the outer protein layer of the BTV virion (Lewis and Grubman, 1990) and mediate the attachment and infection of insect cells (Tan *et al.*, 2001; Xu *et al.*, 1997). The outer diffuse protein layer of the mature virion is composed of 60 VP2 trimers (coded for by segment 2) that are interspersed with 120 VP5 trimers (coded for by segment 6) (Hewat *et al.*, 1992). Viral protein 2 functions as the mammalian cell receptor ligand and contains the epitopes that determine virus serotype (Hassan and Roy, 1999; Huismans and Erasmus, 1981; Kahlon *et al.*, 1983; Mertens *et al.*, 1989). Viral protein 5 functions as the viral fusion protein and facilitates the penetration of viral cores into the cytoplasm of mammalian cells during receptor-mediated endocytosis (Forzan *et al.*, 2007; Forzan *et al.*, 2004; Hassan *et al.*, 2001; Zhang *et al.*, 2010). The protein also appears to play a minor role in influencing BTV serotype, possibly through stearic interaction with VP2 at the virion surface (Cowley and Gorman, 1989; Mertens *et al.*, 1989).

Viral transcription is facilitated by ten transcriptase complexes that are situated on the inner side of the sub-core, below pores that are present at the 5' fold axis of the VP3 icosahedron (Nason *et al.*, 2004). The transcriptase complexes are composed of three minor structural proteins that include VP1 (coded for by segment 1) that functions as the viral RNA-dependant RNA polymerase (Boyce *et al.*, 2004; Urakawa *et al.*, 1989), VP4 (coded for by segment 4) that caps and methylates newly synthesized mRNA strands (Martinez-Costas *et al.*, 1998; Ramadevi *et al.*, 1998) and VP6 that unwinds and re-anneals the dsRNA genome during transcription (Stauber *et al.*, 1997).

Non-structural protein 1 (coded for by segment 5) forms characteristic tubular structures in the

cytoplasm of infected mammalian and insect cells. The exact role that these structures play in the replication cycle of the virus has not been determined (Huismans and Els, 1979; Owens *et al*., 2004; Urakawa and Roy, 1988). It has however been proposed that NS1 may play a role in the morphogenesis of BTV and the intracellular trafficking of the virus to the plasma membrane (Eaton *et al*., 1988). There is also evidence to suggest that NS1 may influence the pathogenesis of BTV infection in the vertebrate host. The interruption of NS1 tubule formation through the expression of anti-NS1 antibody Fc fragments in mammalian cell culture for example has been shown to convert viral release from a lytic infection to non-cytopathic budding and resulted in a significant increase in the amount of virus that was released from infected cells (Owens *et al*., 2004).

Viral assembly is facilitated by the NS2 phosphoprotein (coded for by segment 8) that rapidly accumulates as viral inclusion bodies (VIBs) in the cytoplasm of infected cells (Brookes *et al*., 1993; Modrof *et al*., 2005).It has been demonstrated by immuno-staining that VIBs in addition to NS2 also contains the other structural proteins that are required for core assembly (Brookes *et al*., 1993). The protein is also able to bind to single stranded RNA (ssRNA) and is thought to mediate the assortment of the correct genome segments into progeny viral pre-cursor particles. This process may be mediated by the specific recognition of secondary ssRNA structures by the protein (Lymperopoulos *et al*., 2006).

The intracellular trafficking and release of BTV from infected cells is mediated by the viral glycoprotein NS3 (coded for by segment 10) and its alternative form NS3/A. The NS3/A proteins are integral membrane proteins that are over expressed in insect cells relative to mammalian

cells (Bansal *et al*., 1998; Guirakhoo *et al*., 1995). The proteins are associated with smooth intracellular vesicles as well as the plasma membrane (Wu *et al*., 1992) and co-localizes at sites of viral release at the cell surface (Hyatt *et al*., 1991). Cell lysis with an associated cytopathic effect is mainly observed in mammalian cells, whereas budding of virions without cell death occurs in insect cells (Han and Harty, 2004; Hyatt *et al*., 1993). Cell lysis is likely mediated by two viroporin-like hydrophobic domains that are able to interact with the mammalian cell membrane to induce membrane pore formation (Han and Harty, 2004). The budding of progeny virus particles in contrast appears to be facilitated through the simultaneous interaction of VP2 and NS3/A with components of the host cell export machinery. The N-terminal domain of the NS3/A protein has been demonstrated to be able to interact with the calcipitin light chain (p11) of the cellular annexin II complex that is involved in cellular exocytosis (Beaton *et al*., 2002) as well as Tsg101 a cellular protein that has been implicated in the intracellular trafficking and release of a number of enveloped viruses (Wirblich *et al*., 2006) The C-terminal domain of the protein in contrast is able to bind to VP2 on the surface of mature virus particles, suggesting that the NS3/A protein is able to act as a bridge between the virus and the cellular export machinery (Beaton *et al*., 2002). Studies with mutants, obtained by reverse genetics that express either full length NS3 or NS3A has demonstrated that whereas NS3/A is dispensable for viral release in mammalian cells, both NS3 and NS3/A appear to be required for efficient viral release in insect cells (Celma and Roy, 2011).

The non-structural protein (NS4) has only recently been discovered and only cursory studies have been conducted to elucidate its function in the viral replication cycle. Non-structural protein NS4 appears to localize in the nucleolus of infected cells early during the viral

replication cycle and contains "coiled-coil" domains that are similar to nucleic acid binding proteins (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011). Nucleic acid protection assays have demonstrated that NS4 is able to bind to double stranded DNA (Belhouchet *et al.*, 2011) and it has been suggested that the protein may be involved in subverting host cell antiviral defences, possibly by modulating the transcription of genes in the nucleolus (Ratinier *et al.*, 2011). Indeed it has been demonstrated that NS4 is able to confer a replication advantage to wild type BTV-8 in infected mammalian cells that are in an interferon induced anti-viral state in contrast to a BTV-8 mutant lacking the ability to express the protein (Ratinier *et al.*, 2011).

Bluetongue virus evolution

Bluetongue virus evolves through a combination of genetic drift/shift as well as intragenic recombination (Bonneau *et al.*, 2001; He *et al.*, 2010; Samal *et al.*, 1987a; Samal *et al.*, 1987b; Stott *et al.*, 1987). These evolutionary processes result in the generation of quasispecies populations in the host or vector, from which variants with optimal fitness may be selected under different environmental conditions. Quasispecies evolution together with the random fixation of quasispecies variants during the transmission of the virus between its vertebrate and invertebrate host, appear to be the main mechanisms leading to the observed genetic diversity amongst BTV field strains (Balasuriya *et al.*, 2008; Bonneau and Maclachlan, 2004; Bonneau *et al.*, 2001).

RNA viruses lack a proofreading function in their RNA dependant RNA polymerases and are therefore characterized by an error prone replication process and high mutation rates (Bonneat et al., 2001). *In silico* analysis of segments 2, 3, 6 and 10 of time stratified nucleotide sequences

of a worldwide panel of BTV isolates has shown that the evolutionary rate in these segments vary from between 0.52 to 6.0 x 10^{-4} nucleotide substitutions per site/per year (Carpi *et al.*, 2010). The occurrence of genetic drift has experimentally been confirmed for BTV during the transmission of the virus between its invertebrate and vertebrate host. The sequential transmission of plaque purified BTV-10 between sheep, cattle and *C. sonorensis* midges and the genetic characterization of viral genes (VP2 and NS3 encoding genome segments) of quasispecies variants, indicated that whereas the BTV-10 consensus sequence remained largely conserved, viral replication in each host also resulted in the establishment of an unique set of minor quasispecies variants. It was furthermore also demonstrated that minor variants may be randomly fixed from a quasispecies population by founder effect (genetic bottleneck). One group of *C. sonorensis* that were fed on an infected sheep demonstrated the fixation of a minor variant from the quasispecies population present in the blood of the infected animal (Bonneau *et al.*, 2001).

Genetic reassortment (genetic shift) has long been implicated as a mechanism that can generate genetic diversity amongst RNA viruses with segmented genomes and has been demonstrated for different RNA virus families including *Reoviridae* (Wenske *et al.*, 1985) e.g. Rotavirus (Kirkwood, 2010) and *Bunyaviridae* (Bishop and Beaty, 1988) amongst others. The segmented nature of the BTV genome allows the virus to freely exchange its genome segments between different co-infecting strains in concurrently infected host cells. The opportunity for BTV to reassort in nature is emphasized through the observation that concurrent infections with more than one strain of ruminants in the field has frequently been demonstrated (Brenner *et al.*, 2010; Stott *et al.*, 1982; Stott *et al.*, 1985). Evidence for the occurrence of BTV genetic

reassortment has also been obtained though oligonucleotide fingerprint analysis (Sugiyama *et al.*, 1982) as well as hybridization (de Mattos *et al.*, 1991) and molecular sequencing studies (Batten *et al.*, 2008; de Mattos *et al.*, 1996; Maan *et al.*, 2012a; Maan *et al.*, 2012b; Maan *et al.*, 2010; Mecham and Johnson, 2005; Pierce *et al.*, 1998; White *et al.*, 2006) on BTV field isolates.

The kinetics of BTV genetic reassortment has further been investigated experimentally in sheep (Samal *et al.*, 1987b), bovine (Oberst *et al.*, 1987) and *C. variipennis* (El Hussein *et al.*, 1989; Samal *et al.*, 1987a) as well as in cell culture (Ramig *et al.*, 1989). These studies have indicated that reassortment occurs at varying frequencies in the different host systems. In sheep, the fraction of reassortant progeny clones recovered from viraemic animals was approximately 5% (Samal *et al.*, 1987b), whereas in a similar study in bovine the ratio of recovered reassortant progeny was significantly higher (89%) (Stott *et al.*, 1987). A high fraction of reassortants have also been recovered from mixed infected *Culicoides* midges (7-78% of clones recovered per infected midge). The high proportion of reassortants that have been recovered from concurrently infected midges suggests that the insects are highly permissive hosts for the reassortment of the virus in nature (Samal *et al.*, 1987a). This is especially significant when one considers that *Culicoides* are infected with the virus for the duration of their adult life span and that adult female *Culicoides* may take multiple blood meals (Mellor *et al.*, 2000). The frequency of reassortment in infected African Green Monkey Kidney (Vero) cells has also been shown to be high, with 54% of recovered progeny virions being reassortants (Ramig *et al.*, 1989).

With regards to the type of reassortants that were isolated from different host systems, typically one of the parental strains dominated the yield of recovered virions with the other

parental strain only being represented by its contribution of genome segments to the reassortant progeny. Multiple unique reassortant strains were further isolated from each of the host systems, with some strains dominating the yield of the recovered reassortant progeny later during infection. This suggests that these viruses had either reassorted their genome segments earlier during the infection cycle and had therefore replicated to a higher titre or that the viruses had acquired genome segments that conferred a selective growth advantage over the other reassortant progeny. Indeed reassortment appears to occur non-randomly for some of the genome segments (e.g. 5, 7, 8, 9 and 10) (El Hussein *et al.*, 1989; Ramig *et al.*, 1989; Samal *et al.*, 1987a; Samal *et al.*, 1987b). The exact advantages that are conferred on the reassortant progeny by the selection for or against these genome segments are however unknown.

The asynchronous infection of *Culicoides* as well as vertebrate cell culture with two or more strains of BTV has also shown that cells that have been infected with a particular strain become increasingly resistant against infection with a secondary strain at increasing time intervals post infection, a phenomena that is called "viral exclusion". Although the exact mechanism of viral exclusion has not been determined, it is thought to limit the frequency with which reassortment occurs in the vertebrate host and insect vector to times when these hosts are infected simultaneously with more than one strain of the virus, or nearly so (El Hussein *et al.*, 1989; Ramig *et al.*, 1989).

The exact molecular mechanism that facilitates BTV genetic reassortment has not been determined. It has been suggested that reassortment may be facilitated by the selection of viral

ssRNA by VIBs (NS2) during the packaging of the viral genome segments into sub-core particles (Gould and Hyatt, 1994). The NS2 protein rapidly forms as a matrix around individual transcribing viral cores in the cytoplasm of infected cells. Smaller VIBs eventually fuse to form large cellular inclusion bodies (Fu, 1995), thereby creating the ideal situation for reassortment to occur by bringing genome segments from different virus strains into close association.

Intragenic recombination whereby mosaic genes are generated from the "splicing" together of homologous genes from different ancestral viral strains has recently been demonstrated for BTV. Analysis of 690 complete BTV gene sequences available on Genbank, representing all of the genome segments of the virus has indicated that up to 1.6% of the analyzed sequences represented unique recombination events (He *et al.*, 2010). Recombination was found to have occurred in all of the viral genome segments except segments 5 and 6, with recombinants having undergone either single, double or multiple cross-over events. Interestingly it was found that several BTV strains isolated at different time points and from different geographical locations contained genome segments that appeared to be descended from common mosaic ancestors indicating that recombinant genes had become fixed amongst dominant strains in the field. This observation suggests (but does not prove) that the acquisition of the recombinant genes by these dominant field strains may have resulted in an increased evolutionary fitness of these viruses in the field (He *et al.*, 2010).

The exact mechanism through which BTV recombination occurs is currently unclear. He *et al.*, (2010) suggested that recombination may be facilitated by regions within the viral genome that are prone to the formation of RNA secondary structures. These regions could allow parenteral

RNA segments from disparate origins to interact with each other, thereby allowing recombination to occur. In the study by He *et al*., (2010) secondary RNA structure prediction indicated that the regions around the breaking points of all mosaic sequences demonstrated the potential for RNA secondary structures to form (He *et al*., 2010).

Potential molecular determinants that may influence the phenotype of BTV

The nucleotide sequences of the genome segments of BTV display varying degrees of conservation that reflect both the error prone replication process and the selective pressures that act on the encoded proteins (Balasuriya *et al*., 2008; Bonneau and Maclachlan, 2004; Bonneau *et al*., 2001). In general the genes encoding the proteins of the BTV sub-core (segments 1, 3, 4 and 9) and non-structural proteins (segments 8 and 9) are conserved, due to either functional or structural constraints that act to limit the evolution of the encoded proteins. In contrast, the outer capsid proteins (VP2 and VP5) are exposed to external selective pressures such as the ruminant immune system and the encoding genes are therefore more variable (Maan *et al*., 2008; Maan *et al*., 2007a). Viral protein 7 is relatively conserved within the BTV serogroup, however the encoding gene also demonstrates sequence diversity that clusters the virus into several clades, the biological relevance of which is currently unknown (Bonneau *et al*., 2000; Wilson *et al*., 2000). Similarly the NS3/A encoding gene demonstrates significant nucleotide sequence variability when compared to the segments that encode the other non-structural proteins (Balasuriya *et al*., 2008).

When considering variation in phenotype between BTV strains, differences in virulence or transmission potential are likely attributable to genetic variation within the more variable

genome segments (those encoding VP2, VP5, VP7 and NS3/A). This hypothesis is supported by several studies on BTV and the closely related AHSV that have highlighted the role that changes in these proteins may have on the phenotypic properties of the virus.

Initial evidence for the involvement of the outer capsid proteins in influencing virulence was obtained from cross-hybridization studies that indicated that the proteins undergo changes during attenuation of the virus in cell culture (Huismans & Howell, 1973). The role of VP2 in determining BTV virulence was subsequently investigated in several studies using virulent and non-virulent field strains that indicated that the attenuation of virulent strains may be related to amino acid changes in the VP2 protein that abrogates the ability of the virus to be neutralized by monoclonal antibodies (Bernard *et al.*, 1996; Bernard *et al.*, 1994; Gould and Eaton, 1990; Wilson *et al.*, 2008). Changes in VP2 may however not singly be responsible for differences in the neutralization characteristics of BTV, as it has been demonstrated that amino acid changes in VP5 (without accompanying changes in VP2) may also lead to the development of neutralization resistance (DeMaula *et al.*, 2000).

The role of VP5 in determining the virulence of BTV is supported through the study of two naturally occurring reassortants of BTV-11 named UC-2 and UC-8 that were isolated from the field in the USA. The two viral strains shared the same VP2 segments, but differed with regards to their VP5 segments. The UC2 strain derived its genome segment 5 from a BTV-11 modified-live virus vaccine (MLV) strain, whereas UC-8 derived its segment 5 from a BTV-10 MLV vaccine strain (de Mattos *et al.*, 1991; Osburn, 1994). When inoculated into new-born mice, UC-2 was found to be virulent only when inoculated via the intra-cranial route, whereas UC-8 was

virulent when inoculated via either the sub-cutaneous or intra-cranial route (Waldvogel *et al*., 1987; Waldvogel *et al*., 1986). The two viral strains also differed with regards to their ability to cause disease in foetal calves, depending on the gestational age at which the foetus was infected. When foetuses were inoculated through the uterine wall at 120 days of gestation, both strains were able to cause neurological abnormalities (Waldvogel *et al*., 1992b). In contrast, the inoculation of foetuses with UC-2 at 243 days of gestation led to the birth of healthy calves, while inoculation with UC-8 led to the premature birth of small and weak calves that displayed mild encephalitis (Waldvogel *et al*., 1992a). The underlying molecular basis for the enhanced virulence of UC-8 relative to UC-2 is not known. It has been suggested that it may be related to stearic interaction between the different versions of VP2 and VP5 at the virion surface that could potentially affect both viral binding as well as neutralization (Carr *et al*., 1994).

The precise molecular mechanism that leads to the attenuation of wild type strains following sequential passage in embryonated chicken and cell culture has not been clearly characterized. A study was conducted in order to further investigate the viral genes and amino acid substitutions that are involved in the attenuation of BTV field strains during the virus's passage in atypical host systems (Caporale *et al*., 2011a). In this study the authors compared the virulence of both low and high cell culture passage BTV strains from Italy and South Africa by infecting 3-day old NIH-Swiss and adult interferon receptor deficient mice. In general the low passage field strains demonstrated a virulent phenotype in the murine models, whereas high passage strains were non-virulent. Sequence analysis of the VP1, VP2 and NS2 encoding genome segments demonstrated consistent non-synonymous amino acid changes between low

and high passage virus strains, suggesting that one or more of these proteins may be involved in modulating virulence (Caporale *et al.*, 2011a).

Variation in the NS3/A protein may also potentially influence the severity of BTV infection in the mammalian host as the protein causes cell membrane damage and thus has a significant role in inducing viral cytopathic effect (Han and Harty, 2004). Indeed it has been demonstrated that variability in the NS3/A gene of the closely related African horse sickness virus (AHSV) is associated with differences in cellular cytopathogenecity. The AHSV NS3/A protein in contrast to BTV is highly diverse (37% amino acid sequence variability) and clusters into three phylogenetic clades (α, β and γ) with virus strains belonging to each of these clades differing with regards to the degree with which they are able to induce viral cytopathic effect. The generation of reassortants between parental viruses containing NS3/A genes from each of these clades has shown that the reassortment of NS3/A can significantly alter the cytopathogenecity of reassortant progeny. In this case the extent of these changes was dependant on the type of NS3/A encoding gene that was donated by a particular parental strain (Meiring *et al.*, 2009).

The association of the AHSV NS3/A encoding gene with virulence has been further investigated in the vertebrate host by O'Hara *et al.*, (1998). The authors generated reassortant viruses between a virulent AHSV-3 and a non-virulent AHSV-8 strain dual infection of BHK-21 cells. This yielded two reassortant AHSV-8 viruses that differed with regards to only their NS3/A genome segments. The two reassortant viruses designated as AHSV-A79 and AHSV-A790 contained the NS3/A genes from the avirulent AHSV-8 and virulent AHSV-3 strains respectively and

demonstrated an avirulent and intermediate virulent phenotype in newborn mice. Therefore the genetic variant of the NS3/A encoding gene from the virulent AHSV-3 was able to confer a novel virulence phenotype to non-virulent AHSV-8 (O'Hara *et al*., 1998). Cumulatively these studies suggest that AHSV virulence resides with the NS3/A encoding genome segment. It is therefore interesting to speculate that the NS3/A encoding gene of BTV may similarly play a role in modulating virulence.

Infection and the transmission of BTV by *Culicoides* are influenced by a combination of midge, environmental and virus-specific factors. These factors include amongst others the presence of heritable barriers that prevent the dissemination of the virus in the insect (Fu *et al*., 1999) and ambient temperature that influences the rate of virogenesis and length of the extrinsic incubation period (Wittmann *et al*., 2002). With regards to intrinsic virus specific factors that may influence infection and transmission, the VP7 and NS3/A proteins of BTV play an important role in mediating the infection and dissemination of the virus in the insect vector. It has therefore been considered that genetic variation in these proteins may relate to the transmission of the virus by different midge populations in different geographic locations (Bonneau *et al*., 2000; Maan *et al*., 2008; Maan *et al*., 2010; Wilson *et al*., 2000). The VP7 encoding gene can be divided into several different clades by phylogenetic analysis, however only a weak correlation between these clades and particular geographic locations and/or *Culicoides* populations have been established (Bonneau *et al*., 2000; Wilson *et al*., 2000). Similarly analysis of the amino acid sequence of the NS3/A gene of a global panel of BTV isolates failed to demonstrate adaptive (positive) selection that may be indicative of the co-evolution of this protein with particular *Culicoides* (Balasuriya *et al*., 2008).

Despite evidence of a lack of co-evolution of BTV NS3/A with particular *Culicoides*, one study with AHSV has suggested that the protein may be involved in modulating transmission potential. In this study the effect of variation in the NS3/A gene of reassortant AHSV-A79 and AHSV-A790 (O'Hara *et al*., 1998) to influence infection and dissemination of the virus in *Culicoides* was investigated (Riegler, 2002). Results from the study indicated that *Culicoides* that had been fed on the two reassortant viruses demonstrated differences in both total as well as disseminated infection rates. Overall the results suggested that the two genetic versions of the NS3/A protein either affected the ability of the virus to establish a localized midgut infection or the dissemination of the virus to the insect salivary glands (Riegler, 2002).

Finally bluetongue virus field strains may potentially differ in their infectivity for particular *Culicoides* based on the amino acid sequence of VP2. It has previously been demonstrated that the cleavage of the virus outer capsid protein VP2 with chemotrypsin and N-lauroyl sarcosinate results in the generation of infectious sub-viral particles (ISVPs) with an enhanced infectivity for *Culicoides* cells (Mertens *et al*., 1996). It has recently been demonstrated that a protease in the saliva of *Culicoides* is similarly able to cleave VP2, resulting in the generation of ISVPs. The protease differed in its ability to cleave the VP2 protein of viruses from either eastern or western origins. Interestingly it was also demonstrated that the amount of the protease differed in the saliva of *C. sonorenis* and *C. nubeculosus* midges, a vector competent and non-competent species respectively(Darpel *et al*., 2011).

Towards identifying the molecular determinants that influence BTV phenotype

The characterization of the genetic differences that exist between phenotypically distinct BTV field strains requires high quality sequence data of the whole viral genome. Whole genome amplification strategies that allow the entire BTV genome to be amplified to a high concentration (required for sequencing) in a single reaction tube, as well as the development of high throughout put sequencing technologies have been established (Maan *et al.*, 2007b; Potgieter *et al.*, 2002). Sequence data of the entire BTV genome can now directly be obtained from blood or tissue samples, without prior virus isolation (Potgieter *et al.*, 2002).

Recently a reverse genetics system for BTV was demonstrated through which infectious virus is entirely recoverable by transfecting cells with *in vitro* transcribed ssRNA of each of the viral genome segments (Boyce *et al.*, 2008). This method allows individual viral genome segments to be modified by site directed mutagenesis prior to transfection in order to introduce defined mutations into any region of the viral genome. Genome segments from different parenteral viral strains may also be transfected in different combinations in order to yield defined reassortant genotypes (van Gennip *et al.*, 2012; van Gennip *et al.*, 2010). By using these methods it has become possible to generate "tailor-made" BTV mutants with a defined genetic background. Such viruses have become important tools for the study of the effect of mutation or reassortment in conferring a particular phenotype to the virus.

Several *in vivo* and *in vitro* approaches may prove useful for comparing the effect of mutation on BTV phenotype. In order to evaluate differences in virulence between BTV strains, experimental transmission studies with BTV in ruminants have frequently been used. It is for example common practice to evaluate the degree of attenuation of MLV strains by comparing

disease-specific parameters (temperature, length of viraemia and severity of oral lesions) in vaccinated and unvaccinated animals following virus challenge (OIE, 2004). Transmission studies in live animals have obvious disadvantages for ethical, animal welfare and economic reasons; however transmission studies in ruminants are also subjected to several other disadvantages. The clinical signs of BT may be highly variable between different host species and individual animals (Verwoerd and Erasmus, 2004). Due to this variability, a comparison of virulence between BTV strains by using clinical reaction indexes or by comparing pathological features may not give an accurate reflection of strain-specific differences. Furthermore these studies are expensive to conduct and require the use of a large number of animals as well as specialized vector-free facilities.

The use of small animal models provides a suitable alternative for comparing differences in BTV virulence. It has been demonstrated by immunoperoxidase staining that 11-day old chicken embryos show a similar tissue tropism for AHSV as infected horses (Maartens, 2010). Similarly type 1 interferon receptor deficient mice are highly susceptible to BTV infection via both the oral and intravenous route and demonstrate similar clinical and pathological features as the ruminant host (Calvo-Pinilla *et al.*, 2010; Calvo-Pinilla *et al.*, 2009; Caporale *et al.*, 2011b). The use of *in vitro* methods to compare BTV phenotype also shows promise. The cultivation of primary lung micro-vascular and pulmonary artery endothelial cells from sheep and cattle has made it possible to compare some of the cellular and immunological features that may be responsible for differences in the clinical presentation of BTV in these host species (DeMaula *et al.*, 2001; DeMaula *et al.*, 2002a; DeMaula *et al.*, 2002b). Changes in trans-endothelial cell monolayer electrical resistance (TER) have furthermore been used to provide a quantitative

measurement of the degree of cell monolayer destruction following BTV infection (Drew *et al.*, 2010). It can be envisaged that these parameters may be compared in permissive cell lines that have been infected with different BTV strains in order to illustrate strain specific differences in BTV phenotype.

Culicoides infection models are used for a comparison of differences in transmission potential. Resistance to BTV oral infection appears to be under control of a single genetic locus (Tabachnick, 1991) and progressive inbreeding of *C. sonorensis* midges under laboratory conditions has led to the establishment of susceptible midge colonies that are useful for oral susceptibility studies (Jones and Foster, 1974). The maintaining and feeding of *Culicoides* in the laboratory is well established; however in practice the establishment of laboratory colonies from field caught insects has proven to be an arduous and difficult task. Due to these difficulties only a few species have successfully been colonised (Veronesi *et al.*, 2009). Nevertheless the use of either field caught insects or laboratory colonies have proven invaluable in the investigation of the parameters that influence the transmission of the virus.

Studies on BTV transmission potential are based on the oral infection of *Culicoides* by using blood meals that are spiked with a defined titre of virus, prior to a comparison of either total or disseminated infection rates after the extrinsic incubation has been completed (Carpenter *et al.*, 2006; Venter *et al.*, 2006; Venter *et al.*, 1998). This provides information on the rate with which different midge species or population can be infected with a particular BTV strain and can also give an indication as to how successfully the virus can be transmitted during subsequent biting activity. In the latter case an individual insect's ability to transmit the virus

can be evaluated by demonstrating infection of the insect salivary glands by using either immunohistochemical staining, virus isolation or PCR on the head section of the insect. Alternatively it has been suggested that certain threshold titres of virus in infected midges are indicative of vector competence ($>2.9 \log_{10} TCID_{50}$/midge) (Baylis *et al.*, 2008). In the latter case it should be emphasized that the relationship between the virus load and the efficiency with which the virus is transmitted is still unclear (Carpenter *et al.*, 2008). Other potential pitfalls of oral susceptibility studies in *Culicoides* include difficulties in standardizing the titre of virus in the blood meal prior to blood feeding (Venter *et al.*, 2006), the generally low oral susceptibility of *Culicoides* for BTV (Bellis *et al.*, 1994; Gerry *et al.*, 2001), as well as the potential varying oral susceptibility of field caught *Culicoides* based on their geographic origin (Carpenter *et al.*, 2006; Jones and Foster, 1978; Venter and Paweska, 2007).

Why is it important to identify the molecular determinants that influence BTV phenotype?

The ability to discriminate virulent from non-virulent field strains and to identify viruses with the potential for rapid spread and overwintering represents critical information for informing policy makers with regards to the impact that new outbreaks of the disease are likely to have and for conducting cost-risk analysis prior to the implementation of control measures. This can clearly be demonstrated by the 2006-2008 BTV-8 outbreak in northern Europe (Maan *et al.*, 2008) and the 2008 detection of a BTV-6 MLV vaccine strain reassortant in the eastern Netherlands and later adjacent parts of Germany (Eschbaumer *et al.*, 2009; Maan *et al.*, 2010; van Rijn *et al.*, 2012).

The 2006-2008 outbreak of BTV-8 in northern Europe represented an unprecedented step change in the epidemiology of the virus. This was the first time that BTV had been encountered in northern Europe in a region that was traditionally thought to be beyond the northern most limits where climatic conditions could sustain a bluetongue epidemic (Purse *et al.*, 2005). Several features of the BTV-8 outbreak made it unusual when compared to outbreaks of BT that occur in other regions of the world. The BTV-8 strain was highly virulent and caused acute disease not only in sheep, but also in cattle and goats (Backx *et al.*, 2007; Darpel *et al.*, 2007). The BTV-8 strain also demonstrated the ability to cross the ruminant placenta a (Backx *et al.*, 2009; Darpel *et al.*, 2009; De Clerq *et al.*, 2008; Desmecht *et al.*, 2008; Menzies *et al.*, 2008; Saegerman *et al.*, 2010; van der Sluijs *et al.*, 2011; Vercauteren *et al.*, 2008), a property that had previously only generally been associated with the vaccination of pregnant animals with MLV strains (Flanagan and Johnson, 1995; Parsonson *et al.*, 1987; Schultz and Delay, 1955).. The BTV-8 strain also appears to have had an increased tendency to be secreted in the semen of infected rams and bulls. Two studies have demonstrated the presence of the BTV-8 RNA and/or infectious virus in the semen of naturally infected bulls and rams by using PCR and/or virus isolation (Leemans *et al.*, 2011; Vanbinst *et al.*, 2010). In one of the studies 75-100% of semen samples collected from rams between 25-116 days post observation of clinical signs still tested positive for the virus by PCR (Leemans *et al.*, 2011). Finally the BTV-8 strain was spread by indigenous northern European *Culicoides* species (particularly *C. obsoletus* and *C. pullicaris*) that have not previously been implicated in the widespread transmission of the virus (Meiswinkel *et al.*, 2008).

With regards to the European outbreak of BTV-8, the number field cases was only reduced in Europe in 2008 following widespread vaccination using inactivated vaccines that had only become available that year. The development, testing and authorization of BTV-8 inactivated vaccines were initially delayed in Europe due to uncertainty that existed on the part of pharmaceutical companies with regards to the use of BTV-8 inactivated vaccines in the field (Wilson and Mellor , 2009). In this case knowledge on the genetic determinants that influence BTV virulence could have justified the earlier development and use of inactivated vaccines in order to reduce ruminant mortality. Secondly, European Union member countries still allowed the importation of seropositive pregnant ruminants during the vector free seasons of 2006-2007 before it became clear that the virus was able to cross the ruminant placenta. This approach failed when it was demonstrated that BTV-8 seropositive but PCR negative heifers imported into Northern Ireland from the Netherlands gave birth to either PCR positive and/or viraemic offspring (Menzies *et al*., 2008). This highlighted the possibility of introducing the virus into BTV free countries by using established import/export procedures. Detailed knowledge of the molecular determinants that allow the virus to cross the placenta (such as the genetic markers that affect cell tropism) may have allowed for a timely change in import/export policy before the opportunity had arisen for human assisted dissemination of the virus. In this case a detailed investigation of the genetic changes that leads to an increase in the ability of MLV vaccine strains to cross the placenta as compared to wild type strain may have been informative. Finally a clear understanding of the molecular determinants that influence the efficiency of the transmission of the virus by *Culicoides* may have emphasised the potential for the rapid spread of the virus as was observed during its re-emergence in 2007.

In 2008 the circulation of a BTV-6 MLV strain was detected for the first time in north-western Europe in the eastern Netherlands and later in adjacent parts of Germany (Eschbaumer *et al.*, 2009; Maan *et al.*, 2010; van Rijn *et al.*, 2012). Whole genome sequencing confirmed that the majority of genome segments of this strain were closely related to the South African BTV-6 MLV strain, however the analysis also revealed that the virus had received its segment 10 (NS3/A) from a South African BTV-2 MLV strain (Maan *et al.*, 2010). Although it has been established from field observations and experimental transmission studies in sheep and cattle that the strain was relatively avirulent (Eschbaumer *et al.*, 2009; van Rijn *et al.*, 2012), during its initial emergence it was unknown whether the virus would have any significant animal health or economic impact and whether the virus would demonstrate the capability to overwinter. It is frequently highlighted in the literature that genetic reassortment between distinct BTV strains may result in the generation of viruses that demonstrate unique biological properties (Batten *et al.*, 2008; Brenner *et al.*, 2011; Maan *et al.*, 2010; Saegerman *et al.*, 2008; Veronesi *et al.*, 2005). The risk that genetic reassortment poses with regards to the alteration of viral phenotype has however not thoroughly been investigated, with only a few studies available that have directly addressed the issue for BTV. When a wild type-vaccine reassortant BTV emerges in the field such as occurred in northern Europe, it thus initially remains an open question as to whether the virus will demonstrate unique properties. In this case a clear understanding of the determinants influencing the phenotype of BTV may have been useful for predicting the threat that the reassortant may have posed to the European livestock industry.

Competing interests

The author(s) declare that they have no competing interests.

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