Recent advances in knowledge of BTV-host-vector interaction

Peter Coetzee* & Estelle H. Venter

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa.

* *Corresponding author at: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa. Tel.: +27 12 529 8269, e-mail: peter.coetzee@yahoo.com.*

Veterinaria Italiana 2015, **51** (4), 301-311. doi: 10.12834/VetIt.490.2367.1
Accepted: 31.01.2015 | Available on line: 31.12.2015

IV International Conference on *Bluetongue and Related Orbiviruses*. November 5-7, 2014 - Rome, Italy - Selected papers

Keywords
Bluetongue virus,
Culicoides,
Immunology,
Molecular biology,
Pathogenesis,
Vaccines,
Virulence,
Virus-vector-host
interaction.

Summary
Bluetongue virus (BTV) has since 1998 extended its distribution further North than where it has previously been encountered. Changes in the epidemiology of Bluetongue (BT), as well as novel features of recent outbreaks of BTV in Europe, have stimulated research on BTV-vector-host interaction. The outbreak of BTV-8 in Northern Europe from 2006-2008 is particular noteworthy in this regard, as the European strain of BTV-8 demonstrated novel properties, including high virulence – especially for cattle – and the capability to cross the ruminant placenta. The virus was in addition transmitted by indigenous European *Culicoides* species that had not previously been implicated in the widespread transmission of BTV. Recent advances in the scientific understanding of BTV-vector-host interaction include increased knowledge of the virus' replication cycle, the role of biotic factors in influencing viral infection of the insect vector, increased knowledge of BTV immunology and pathogenesis in the mammalian host, and increased knowledge of virulence and pathogenicity features of newly discovered serotypes/strains of the virus. New research on aspects of BTV-vector-host interaction has been driven in part by developments in molecular biology and experimental infection biology, of which next generation sequencing, the expression of individual viral proteins in cell culture, the establishment of a reverse genetics system for the virus, the development of novel *in vitro* and *in vivo* infection models, and refinement of existing BTV experimental infection methodologies have proven instrumental. Moreover, these developments have also provided the opportunity for the development of novel vaccine strategies. This article provides a synopsis of selected recent advances that have been made in the understanding of BTV-vector-host interaction, with a particular focus on research that has been conducted in Europe over the last 5 years.

Gli ultimi sviluppi della ricerca sulle interazioni BTV-vettore-ospite

Parole chiave
Virus della Bluetongue,
Biologia molecolare,
Culicoides,
Immunologia,
Ospite,
Patogenesi,
Vaccini,
Vettore,
Virulenza.

Riassunto
A partire dal 1998 il virus della Bluetongue (BTV) ha esteso i propri confini raggiungendo latitudini a nord mai toccate in precedenza. I cambiamenti di natura epidemiologica osservati durante i focolai di Bluetongue (BT) in Europa del 2006-2008 hanno stimolato in misura crescente le attività di ricerca sulle interazioni BTV-vettore-ospite. Il ceppo europeo di sierotipo 8 del virus della Bluetongue (BTV-8) responsabile dei focolai del 2006-2008 ha infatti evidenziato particolari caratteristiche quali l'elevata virulenza, soprattutto per i bovini, e la capacità di attraversare la barriera placentare. Il ceppo è stato inoltre diffuso da specie di *Culicoides* mai ritenute in precedenza possibili vettori di BTV. I progressi raggiunti dalla ricerca negli ultimi 5 anni hanno ampliato le nostre conoscenza sul ciclo di replicazione del virus, sul ruolo dei fattori biotici nelle infezioni virali degli insetti vettori, sull'immunologia e patogenesi nei ruminanti, sulla virulenza e sulle caratteristiche patogeniche dei nuovi sierotipi/ceppi del virus. In molti casi la ricerca sulle interazioni BTV-vettore-ospite è stata facilitata dagli sviluppi nel campo della biologia molecolare e delle infezioni sperimentali. In particolare, le tecniche di *next generation sequencing* (NGS), la possibilità di esprimere singole proteine virali in colture cellulari, l'applicazione della *reverse genetics*, lo sviluppo di nuovi modelli di infezione *in vitro* e *in vivo* e il perfezionamento degli esistenti protocolli

di infezione sperimentale si sono rivelati fondamentali anche per lo sviluppo di vaccini di nuova generazione. Questo articolo offre un riepilogo degli sviluppi della ricerca degli ultimi anni sulle interazioni BTV-vettore-ospite dando particolare enfasi alla ricerca condotta in Europa negli ultimi 5 anni.

Introduction

Bluetongue virus (BTV) is the prototype of the *Orbivirus* genus in the family *Reoviridae* and the causative agent of Bluetongue (BT), an economically important, *Culicoides*-transmitted, non-contagious, thrombo-haemorrhagic viral disease of domestic and wild ruminants, as well as certain camelid species (MacLachlan 2011). Due to its importance to the global livestock industry, BTV has been the subject of intense scientific study since its discovery and as such represents one of the best-characterized viruses on the molecular and structural level. Nevertheless, fundamental aspects of the virus, its host and vector interaction, are not fully understood. Recent changes in the epidemiology of BTV particularly in Europe since 1998, has stimulated research in the BT field, including research on BTV-vector-host interaction.

Bluetongue virus-vector-host interaction can individually be understood at the level of the virus, the vector, and the host. At the virus level, BTV-vector-host interaction may refer to the replication of the virus in either mammalian or vector cells and includes any associated host-cell protein interactions. The ability of the virus to infect and replicate in either host or vector cells may be influenced by virus encoded virulence factors, that may act to promote viral infection and/or that may inhibit the host or vector's antiviral responses. Virulence factors in turn, are influenced by viral evolutionary processes that may modulate virulence, in order to promote long term persistence of the virus in the environment. At the level of the vector, BTV-vector-host interaction may refer to the interaction of the vector with either the virus or the host. This may include the activity of vector associated viral infectivity factors present in the saliva of *Culicoides* vectors, which may act to modify BTV proteins, thereby promoting infection of the insect or which may suppress the host's immune response. At the host level, the primary considerations as regards to BTV-vector-host interaction includes any direct or indirect pathological effects that may be induced by the virus, as well as general infection kinetics and novel features such as transplacental infection or seminal shedding. Vaccines, particularly those that demonstrate the capability to replicate and/or express proteins in ruminant cells, as well as the interaction of BTV vaccines with the mammalian hosts' immune system, may also be considered as falling within the broad theme of BTV-vector-host interaction.

In this article, new knowledge that has contributed to our current understanding of BTV-vector-host interaction will be discussed. This article will focus on research that has been conducted in Europe over the last 5 years, as arguably the majority of research publications have been generated from the region during this span of time, concomitantly with renewed interest in BTV, following outbreaks of BTV-8 in Northern Europe.

Technical advances that have promoted recent research on BTV-vector-host interaction

Over the last few years, several techniques in BTV research have become main stream and are being used by an increasing number of research groups to study various aspects of BT disease, including BTV-vector-host interaction. An increasing number of studies has deployed next generation sequencing, combined with reverse genetics and site directed mutagenesis along with older technologies, such as baculovirus expression of individual viral proteins in cell culture and the use of the yeast-two hybrid system, in order to investigate such aspects as BTV protein function, the interaction of the BTV proteins with the host cell, and to elucidate the different steps in the viral replication cycle (Mohl and Roy 2014). As for alternate infection models for host and vector studies, there has not been significant recent developments in *in vitro* culture systems for BTV. Although assays to examine specific aspects such as the effect of the virus on cell viability, *i.e.* viral cytopathic effect induction and the mechanisms of cell death that are induced in response to BTV infection, have seen major advancements (Coetzee *et al.* 2014). In particular, novel developments in *in vitro* culture systems (*i.e.,* electrical impedance assays) have been used to investigate alternate BT pathogenesis mechanisms, particularly those that relate to the role of cytokines and pro-inflammatory mediators in influencing endothelial cell dysfunction and vascular permeability (Drew *et al.* 2010). From the perspective of BTV vector infection models, except for the use of *Culicoides sonorensis* and *Culicoides nubeculosis* colonies and field caught

insects that have traditionally been used for BTV vector studies, a major limitation that still exists has been a failure to colonize the old world vector species *Culicoides imicola* as well as any of the known European *Culicoides* vector species. In this regard the continued development of other insect models such as *Drosophila melanogaster* – the latter of which has recently been shown to support BTV replication – may partly fill the gap that exists in the availability of BTV-*Culicoides* infection models (Shaw *et al.* 2012).

Traditionally, newborn mice have been used for BTV isolation and virulence studies; however, newborn mice are logistically difficult to handle, become refractory to infection at more than 2 weeks of age. Furthermore, they are not always susceptible to BTV infection using different inoculation routes. Recently, interferon alpha/beta receptor deficient (IFNAR) mice have been developed as an alternate BTV infection model in order to overcome some of these problems. For example, IFNAR mice are susceptible to BTV at any age and via different inoculation routes, and have thus in recent years seen use in an increasing number of BTV virulence and vaccine efficacy studies (Calvo-Pinilla *et al.* 2009). With respect to infection models in the native mammalian host, there has been a plethora of recent studies exploring BTV-1 and BTV-8 infection in a wide range of domestic and wild ruminant species as well as in cervids and camelids (reviewed by Coetzee *et al.* 2014). These studies can be considered as novel infection models to explore such aspects as host susceptibility, BTV induced clinical signs, infection kinetics and pathogenesis in these different host species. Amongst these studies, descriptions of comparative virulence studies using different serotypes of the virus are worth mentioning (Sanchez-Cordon *et al.* 2013), as well as studies that have explored the effects of co-infection with different serotypes (Dal Pozzo *et al.* 2013).

Concerning developments in BTV experimental infection methodology, recent studies have been published that have explored different routes of inoculation (Umeshappa *et al.* 2011), the use of blood grown versus cell culture inoculums (Eschbaumer *et al.* 2010) and the role of infectious dose in reproducing BT clinical disease (Di Gialleonardo *et al.* 2011). These studies have indicated the utility of the subcutaneous route, the use of either blood or cell culture grown virus, as well the use of variable dosing regimens in reproducing BT clinical disease under experimental conditions.

Molecular biology, host cell interaction and evasion of antiviral defences

Bluetongue virus demonstrates a complex replication cycle that can be divided into several steps that include adsorption, viral uptake, fusion/uncoating, transcription, protein translation, core assembly, and viral maturation/egress. At each of these steps, the viral proteins interact with each other and protein components of the host cell, and indeed the viral protein domains that facilitate these interactions have been mapped in detail over the last few years (reviewed by Mohl and Roy 2014). Recent studies on BTV have increasingly focused on elucidating the precise role of the non-structural proteins in the virus' replication cycle and their role in mediating viral evasion from host cell antiviral defence mechanisms. In particular, recent studies have indicated a role for NS1 in regulating viral protein translation and have indicated a role for the newly discovered viral non-structural protein 4 (NS4) in subverting the interferon response in mammalian cells.

As regards to NS1, until recently knowledge of the function of NS1 has been limited to a proposed role in influencing the mechanism of virus release (Owens *et al.* 2004). In a recent study by Boyce and colleagues (Boyce *et al.* 2012), by using a green fluorescent and renilla luciferase mRNA reporter system (*i.e.,* the reporter open reading frame linked to NS3a 5' and 3' UTR sequences), it was demonstrated that the co-expression of NS1 leads to the increased expression of the reporter proteins. These findings suggest a role for NS1 in the up-regulation of protein translation from viral transcripts. Linkage of the reporter systems to different viral UTR sequences indicates that the interaction of different viral UTRs from the different genome segments with NS1, leads to different levels of reporter protein expression, thus suggesting a level of control by which translation of the amount of each viral protein in the cell is controlled. Non-structural protein 1 mediated up-regulation of viral protein translation appears to be specific for viral RNAs, as poly A tailing of the 3' end of the reporter mRNAs abrogated the NS1 mediated effect on reporter protein expression. Currently, the exact mechanism by which NS1 is able to increase protein translation from viral transcripts has not been elucidated (Calvo-Pinilla *et al.* 2009).

Bioinformatics analysis indicated as early as 2008 that segment 9 of BTV may potentially encode an additional protein in the +1 reading frame within the VP6 cistron (Sanchez-Cordon *et al.* 2012). Recent studies have confirmed the presence of a new segment 9 encoded non-structural protein, termed NS4, in BTV infected cells (Belhouchet *et al.* 2011, Ratinier *et al.* 2011). These studies indicated that NS4 is highly conserved within the BTV serogroup, that it is expressed in mammalian and insect cells, and that it localizes alternatively in the nucleolus and plasma membrane during early and late infection, respectively. Modification of NS4 of BTV-1 and BTV-8 so that it is no longer expressed in mammalian cells indicate that the protein is dispensable for virus

replication *in vitro*. Whereas, inoculation of IFNAR mice with NS4 negative BTV mutants shows that the protein does not affect virulence, at least in this model of disease. Significantly, studies with NS4 negative BTV mutants indicates that the protein confers a replication advantage to BTV in interferon treated mammalian cells, suggesting that the protein may act to subvert the interferon response (Ratinier *et al.* 2011).

Significant advances have been made in recent years with respect to the understanding of the entry mechanisms that are used by BTV to enter host cells, the mechanisms by which cells are able to sense BTV infection, and the signalling pathways that lead to the induction of innate immunity and cell death. Pertaining to cell entry, Gold and colleagues (Gold *et al.* 2010), recently described a novel mechanism by which baby hamster kidney (BHK) cells may be infected with BTV. This novel entry mechanism occurs independently from clathrin or caveolae mediated endocytosis and shares features with macropinocytosis (Gold *et al.* 2010). As regards to sensing of BTV infection in host cells, 2 recent studies have described the mechanisms by which immune (*i.e.,* plasmacytoid dendritic cells) and non-immune cells (*i.e.,* epithelial cells) are able to sense BTV RNA following infection, and have identified components of the signalling pathways through which BTV RNA sensing leads to the induction of IFN-1 synthesis (Chauveau *et al.* 2012, Ruscanu *et al.* 2012).

The pathways that lead to cell death as well as aspects of the induction of innate immunity in BTV infected cells has also recently been explored in more detail by Stewart and colleagues (Stewart *et al.* 2010). This study indicates that both the intrinsic (caspase-9 activation) and extrinsic pathways (caspase-8 activation) are involved in BTV induced apoptosis in mammalian cells, and that the two pathways function independently of each other. Cleavage of caspase 8 and 9 in BTV infected cells were associated with the activation of executioner caspases 3 and 7 and the cleavage of PARP [poly (ADP-ribose) polymerase], a host protein that is involved in DNA repair. The article also reports that both NFkB and interferon regulatory factors 3 and 7 were translocated to the nucleus in response to BTV infection, suggesting their role inducing an antiviral state (Mortola *et al.* 2004). By using IRF and NFkB dependant luciferase reporter systems it was further shown that the induction of NFkB and interferon regulatory factors occurred early in the cell following BTV infection, and that the action of these mediators could be inhibited by BTV NS1 and NS2. Moreover, except for the specified role of NS1 and NS2 in the inhibition of the IFN response, a recent report has indicated that NS3/NS3a likewise inhibits the RIG-1 like receptor signalling pathway that is involved in IFN-1 synthesis (Chauveau *et al.* 2013).

Virulence factors

The underlying molecular determinants that influence the virulence of BTV have been difficult to pin down, due to the role of host, vector and environmental factors in influencing BT disease severity. It has previously been suggests that variable virulence characteristics of BTV may be associated with changes in viral properties, like viral attachment and penetration. The overall rate of viral replication, the efficiency/mechanism of viral release, together with associated membrane damage, cell death and viral spread, suggesting a role for VP2, VP5 or NS3/NS3a in influencing BTV virulence (Huismans *et al.* 2004). Indeed, earlier cross hybridization studies have indicated a role for variation in VP2 and VP5 in influencing the virulence of wildtype and attenuated BTV strains (Huismans and Howell 1973), whereas variation in African horse sickness virus (AHSV) NS3/NS3a, a closely related orbivirus, has specifically been linked with altered virulence of AHSV reassortant strains in newborn mice (O'Hara *et al.* 1998). Genetic reassortment of VP5 of BTV-11 strains has further demonstrated a role for genetic variation in segment 6 of BTV in influencing the pathogenesis of BTV in newborn mice and foetal cattle (Waldvogel *et al.* 1987, Waldvogel *et al.* 1992). Beyond these studies, virulence markers of BTV have until recently received little attention. However, the observation that the European strain of BTV-8 was virulent for sheep as well as for cattle and goats has stimulated renewed interest in identifying possible underlying virus, host or environmental factors that affect BTV virulence.

The attenuation of BTV vaccine strains has historically been achieved by sequentially passaging BTV in either embryonated chicken eggs or cell cultures. In order to investigate the mutations that are associated with the attenuation of the virus in these atypical host systems, a recent study compared the virulence properties of low and high passage BTV strains in different mice models, and attempted to correlate virulence changes, with changes in the genotype of the virus. Caporale and colleagues (Caporale *et al.* 2011), sequentially passaged virulent Italian field strains of BTV-2, BTV-4 and BTV-9 until attenuation were achieved in NIH and IFNAR mice. In addition, the authors compared the virulence of live attenuated vaccine (MLVs) and wild-type strains of the same serotypes of South African origin, using the same murine models, in which case the virulence properties of the viruses (*i.e.,* attenuated vs. virulent phenotype of vaccine vs. wildtype strains) were maintained, specifically in IFNAR mice. Genomic comparisons of high and low passage Italian field strains as well as wildtype and vaccine strains from South Africa, indicated that attenuation of the viral strains in mice was associated with consistent

non-synonymous mutations of virulent strains in genome segments encoding VP1, VP2 and NS2 (Caporale *et al.* 2011).

A second study (Caporale *et al.* 2014) explored the effects of host species (sheep and goats) and breed, the age of individual animals, viral serotype, prolonged circulation of a virulent BTV-8 strain in the field, and host versus vector cell replication of BTV, on the clinical course of BT. The results of the study indicated that the breed and age of ruminants appear to only play a small role in BT disease expression when compared to, for example, differences in species susceptibility. Little differences in the virulence of viruses from different serotypes (*i.e.,* BTV-2 and BTV-8) were found. Interestingly enough, it was also found that the European strain of BTV-8 decreased in virulence during its circulation in the field. Genomic comparison of virulent BTV-8 isolated at the beginning of the BTV-8 outbreak in Europe and 'more' attenuated BTV-8 isolated towards the end of the outbreak indicated non-synonymous mutations in genes including VP1, VP2, VP4, VP6, NS1, and NS2. An interesting aspect of the latter study was the demonstration that animal and *Culicoides* cell passaged virus showed increased virulence when compared to virus that was passaged on BHK cells. Deep sequencing of the quasispecies population in this case indicated that passage of the virus on BHK cells led to a genetic bottleneck and reduced quasispecies diversity, whereas blood inoculums and insect cell passaged virus demonstrated a high degree of diversity (Caporale *et al.* 2014). Overall, the latter findings suggest that quasispecies diversity, as a whole and perhaps specific mutations within the quasispecies population itself should be considered, when attempting to investigate aspects such as BTV virulence.

The studies by Caporale and colleagues (Caporale *et al.* 2011, Caporale *et al.* 2014), as well as earlier studies that have attempted to elucidate the effect of reassortment of particular genes on BTV phenotype, lay the groundwork for more specific studies to map the mutations that may be involved in influencing BTV virulence. Such information could hypothetically be applied to the development of molecular tests to screen BTV field strains for particular virulence characteristics. Moreover, the potential effects of reassortment and/or recombination of BTV field and/or vaccine strains on the virulence of parental viral strains can only be predicted, if the genetic markers that influence BTV virulence have been identified.

Vector associated viral infectivity factors

Bluetongue virus is almost exclusively transmitted by the bites of *Culicoides* midges. It has previously been demonstrated that vector saliva can influence the infectivity of certain viruses (*e.g.,* West Nile virus, Sindbis virus, and vesicular stomatitis virus) all of which appear to be more infectious in the presence of vector saliva (Darpel *et al.* 2011). Two mechanisms have been proposed by which vector saliva may promote virus transmission. Vector salivary proteins may potentially either modify a virus protein, thereby increasing its infectivity or, alternatively, may play role in modulating the hosts' immune response (*e.g.,* by down regulating the host cytokine response), thereby facilitating virus infection and transmission. Both these hypotheses have been explored for BTV in recent studies.

Regarding the potential effect of vector saliva on the structure and infectivity of BTV, it has previously been demonstrated that treatment of BTV with proteinases such chemotrypsin results in the cleavage of the viral outer capsid protein VP2 and the generation of infectious sub-viral particles (ISVPs). Infectious sub-viral particles, demonstrate enhanced infectivity for *Culicoides* cells *in vitro* (Mertens *et al.* 1996). In a more recent study (Darpel *et al.* 2011), the authors explored specifically the effects of *Culicoides* salivary proteins, on influencing the structure/infectivity of BTV as well Epizootic haemorrhagic disease virus (EHDV) (Darpel *et al.* 2011). In this study, different strains of BTV and EHDV were incubated with either *C. sonorensis* or *C. nubeculosis* saliva, respectively. Incubation of the viruses with saliva from each of these vector species indicated that *Culicoides* saliva, similar to chemotrypsin *in vitro*, can cleave BTV and EHDV to generate ISVPs with enhanced infectivity for *Culicoides* cells. By using mass spectrophotometry, the author identified a 29-kDA trypsin-like protease that was associated with VP2 cleavage. Interestingly, it was found that the saliva of vector and non-vector competent species, *Culicoides sonorensis* and *Culicoides nubeculosis,* respectively, differed in their efficiency to cleave VP2. Saliva of *C. nubeculosis* further differed in its ability to cleave EHDV from eastern and western lineages.

From the perspective of the effect of vector salivary proteins on the induced immune response of the mammalian host, and its possible effect on influencing disease severity and/or virus transmission, only one recent study has been published for BTV (Pages *et al.* 2014). This study explored the effects on the disease severity in sheep of 3 inoculation routes, including BTV-8 inoculation through intradermal needle inoculation, intradermal inoculation at the site of uninfected midge feeding and inoculation through infected midge bites. Significantly, BTV inoculation through infected midge bites was associated with enhanced viraemia and clinical signs, versus the other two routes. Infected midge bites were further associated with reduced inflammation at the

feeding site, delayed IFN-induced gene expression and a retarded neutralizing antibody response. The modulatory effects of infected versus uninfected midge bites raises the possibility of differential salivary gland transcript profiles in infected and uninfected midges that may affect the induced host immune response and disease expression.

Several studies, focusing aspects such as differential gene expression between blood fed and non-blood fed midges or the protein composition of *Culicoides* saliva, have been conducted with the goal of identifying salivary proteins that are involved in influencing orbivirus infectivity or that are involved in influencing the host immune response and/or disease severity. The transcriptome of mid-gut and salivary glands for blood and non-blood fed fed *C. sonorensis* midges was published in 2005 (Campbell *et al.* 2005). Nonetheless, more recently, the protein composition of *C. sonorensis* saliva without background from *Culicoides* salivary gland proteins was specifically studied (Lehiy and Drolet 2014). In this study, *C. sonorensis* midge saliva was collected during the feeding of midges on sucrose solution and analysed by tandem mass spectrophotometry. Forty-five proteins were identified, 25 of which appear to be orthologs unique to *Culicoides* species. This study also confirmed the presence of the 'late' trypsinase implicated in the study by Darpel and colleagues (Darpel *et al.* 2011) in influencing orbivirus infectivity. Due to the large number of unknown proteins with unknown function in the salivary secretome of *C. sonorensis*, the authors of this study suggested that future work should use recombinant techniques to express the proteins individually, in order to further study the role the individual proteins in influencing orbivirus transmission and disease severity.

Pathogenesis in the mammalian host

Two of the main recent advances that have been made in the understanding of the pathogenesis of BT include the elucidation of the role of skin cells in BT infection, as well as the recognition that cytokines, that are secreted by BTV infected cells, besides direct virus mediated injury, contribute to the pathogenesis of BT disease. The role of skin in supporting BTV replication has recently been re-examined by Darpel and colleagues (Darpel *et al.* 2011). In this article, immuno-fluorescence-labelling of BTV non-structural and structural proteins (*i.e.,* NS2 and VP7) together with confocal microscopy, was used to distinguish between virus presence and replication in thick skin sections from sheep. Replication was demonstrated in 2 major cell types: vascular endothelial cells and agranular mononuclear leukocytes. The authors suggested that the role of skin as a major organ for BTV replication *in vivo* and its role in the transmission of BTV to *Culicoides* has up till now been underestimated, due to the inherent problem of extracting viral RNA from skin for molecular testing (Darpel *et al.* 2012).

Except for the role of skin in supporting BTV replication, the recognition of skin conventional dendritic cells (cDCs) in facilitating BTV infection has also been a major development with regards to the understanding of the pathogenesis of BT. One study has addressed in detail the role of ovine cDCs in BTV infection, pathogenesis and the induction of host immune responses (Hemati *et al.* 2009). The authors used lymph cannulation in sheep, combined with different *in vitro* and *in vivo* techniques to explore cDCs function. The results of the study indicated that BTV utilizes cDCs for its transportation to draining lymph nodes from superficial sites of midge feeding at the skin surface, and that lymph cDCs support production of infectious virus of different BTV serotypes regardless of level of attenuation. The study further indicated that BTV infection provokes a massive recruitment of cDCs to skin of sheep and afferent lymph, where they provide cellular targets for infection. Interestingly, BTV was found to productively infect cDCs with no negative impact on their physiology, highlighting the important role of this cell type in facilitating the early stages of BTV infection. The role of cDCs in the induction of innate immunity was confirmed by the observation that cDCs respond to BTV infection by up-regulating surface expression of T-cell co-stimulatory molecules as well as the synthesis of cytokines involved in inflammation and immunity. Bluetongue virus infected cDCs additionally stimulated antigen specific CD4 and CD8 cell proliferation as well as gamma interferon production *in vitro* (Hemati *et al.* 2009).

Previous *in vitro* studies have suggested that the secretion of pro-inflammatory mediators and cytokines by BTV-infected cells, in addition to direct virus mediated damage of BTV susceptible host cells, plays a role in BTV pathogenesis (Drew *et al.* 2010). One recent study has attempted to assess the relative contribution of cytokines versus direct virus mediated injury of host cells in influencing BT pathogenesis *in vivo* in small ruminants. Sanchez-Cordon and colleagues (Sanchez-Cordon *et al.* 2012) examined tissues containing gross lesions from BTV-1 infected goats using immunohistochemical techniques, and evaluated co-staining of virus antigen and selected cytokines (IL-1α and TNF) with vascular lesions. The results from the study indicated that vascular lesions were indeed not always associated with endothelial cell destruction and viral antigen staining, however vascular lesions were frequently associated with staining for the selected cytokines. In some cases vascular lesions were not associated with either significant viral antigen or cytokine staining, which

suggests that the endocrine action of cytokines from distant infection sites, or that are secreted by other cells such as infected monocytes/macrophages, also play a role in the development of vascular lesions (Sanchez-Cordon *et al.* 2012).

Studies on transplacental transmission, overwintering and seminal shedding

An unusual characteristic of the BTV-8 outbreak in Northern Europe was the observation that the virus, similar to modified live virus vaccine (MLVs) strains, was able to cross the bovine and ovine placenta to cause congenital infection and malformation. Efforts to understand the reproductive aspects around BTV-8 infection, has led to an increased understanding of BTV transplacental infection as well as the potential effects of BTV infection on the reproductive performance of ruminants in general. In particular, BTV-8 transplacental infection has been studied extensively in sheep, cattle and goats under both field and experimental conditions (reviewed by Coetzee *et al.* 2014) whereas infection of blastomeres of zona pellucida free bovine and caprine blastocysts has also been demonstrated with BTV-8 *in vitro*. Of particular note as regards to transplacental infection was the finding that transplacental infection led to the birth of relatively low numbers of viraemic offspring (Barros *et al.* 2007). These findings in turn has stimulated research on other potential BTV overwintering mechanisms, including studies on *Culicoides* survival throughout winter (Clausen *et al.* 2009) and the potential role of ticks (Bouwknegt *et al.* 2010) to act as vectors and overwintering hosts for the virus.

Another atypical aspect of BTV-8 infection in Europe was the observation that the virus was secreted at a high frequency in the semen of naturally infected rams and bulls. Previous studies have indicated that BTV can contaminate the semen of BTV-infected rams and bulls, but this was thought to only occur in older animals, in association with infected blood leaking into the reproductive track or that it was a property that was associated with specific strains of the virus (*i.e.,* MLVs) (Kirkland and Hawkes 2004). Studies on BTV-8 from Europe have indicated that the virus was secreted in the semen of rams and bulls of different ages, at a high frequency, and in the absence of blood contamination, which is unusual for a wildtype strain (Leemans *et al.* 2011, Muller *et al.* 2010). Transmission through contaminated bull or ram semen to recipient ewes and/or heifers has not been confirmed for BTV-8, although venereal transmission with other serotypes/strains of the virus has been documented (Kirkland and Hawkes 2004).

Vaccine safety/novel vaccine approaches

Reproductive problems associated with BTV infection have traditionally been associated with vaccination with MLV strains. In recent years the adverse effects associated with the use of MLV strains has been investigated in greater detail. With respect to MLV safety, studies with MLVs in Europe have indicated incomplete attenuation of certain serotypes in European sheep breeds. Vaccination of European breeds for example with MLV-2, MLV-4, MLV-9, and MLV-16, indicated that some strains can cause overt disease and/or cause high viraemias that are sufficient for transmission of the vaccine strains to *Culicoides* (Veronesi *et al.* 2010). Circulation of MLV strains in the field is of potential concern as MLVs may hypothetically revert to virulence via genetic drift, reassortment or recombination with field strains, although supporting evidence for reversion to virulence of BTV MLVs is currently lacking. Worryingly, there are at least 3 reports of BTV wildtype-MLV reassortants that have since 2002 been isolated from Southern and Northern Europe in the field (Barros *et al.* 2007, De Clercq *et al.* 2009).

Recently, Savini and colleagues (Savini *et al.* 2010) investigated the risk factors that are associated with adverse effects (*e.g.,* temperature, oedema, lameness, hyperaemia, and decrease in milk production) of MLV vaccination of sheep and goats in the field in Italy, where MLV strains had previously been used. This study indicated a link between adverse effects and vaccination with particular MLV serotypes such as MLV-16 as well as Maedi-Visna positive serology (Savini *et al.* 2010). A retrospective study conducted on aborted material from ruminants collected during a 2003-2005 vaccination campaign with MLVs in Italy further confirmed that both BTV-2 and BTV-9 MLV strains used during the outbreak were associated with abortions and foetal malformation of sheep, cattle, and goats (Savini *et al.* 2014). Both these studies highlight the dangers of using MLV strains in the field - at least for the European BT situation - and highlight the need for new and improved strategies to vaccinate against BT disease.

Since MLV strains are associated with many adverse effects including incomplete attenuation, possible reversion to virulence and transplacental infection, their use in Northern Europe is not recommended. Several other vaccine strategies have consequently been developed in an attempt to overcome the inherent problems that are associated with MLVs. Inactivated vaccines were instrumental in bringing the European BTV-8 outbreak under control. However, despite their proven efficacy and the long-term protection that these vaccines provide, their costs and the need to booster vaccination still raise problems. In order to address these

issues, several new vaccine approaches have been developed. These include recombinant vaccines using different virus backbones, DNA vaccines and virus like particles (Noad and Roy 2009). Particularly intriguing is the recent development of disabled infectious single cycle (DISC) and disabled infectious single animal vaccines (DISA) that are able to mimic the natural tropism of the virus, and that are able to express BTV proteins at the site of infection. These vaccines may be designed to be DIVA (distinguishing infected from vaccinated animals) compliant. Disabled infectious single cycle vaccine strains typically contain deletions in one or more viral genes that are essential for viral replication (*e.g.,* VP6) and are therefore avirulent, as the strains can only be grown in modified cell cultures in which the disabled viral proteins' function is provided *in trans* (Matsuo *et al.* 2011). Disabled infectious single-animal vaccines in contrast are able to replicate *in vivo*, however due to deletions of virulence determinants (*e.g.,* deletions in the open reading frame of segment 10), demonstrate both an avirulent phenotype and significantly reduced viraemia, making them unlikely to be transmitted in the field (Feenstra *et al.* 2014). Finally, another strategy that offers the potential to rapidly generate inactivated vaccines against emerging serotypes is the use of reverse genetics coupled with synthetic biology (*i.e.,* commercial DNA synthesis) and binary ethyleneimine inactivation. A recent report describes the use of a BTV reverse genetics attenuated backbone that was used as the basis for the generation of inactivated vaccines against up to 16 serotypes of the virus, via VP2 and VP5 reassortment, prior to inactivation (Nunes *et al.* 2014).

Novel features of newly discovered serotypes

Bluetongue virus serotype 25 was discovered in goats in Switzerland in 2008, at which time it was demonstrated that the virus was not pathogenic for goats – in contrast to sheep – where infection was associated with mild clinical signs and BTV associated pathology (Chaignat *et al.* 2009). A feature of the BTV-25 strain has been the failure to isolate it in *Culicoides* or *Culicoides* cell culture, suggesting that the virus may utilise novel transmission mechanisms. Since that time, there have been several additional studies that have explored the virus' transmission routes. In one study, experimentally infected pregnant goats failed to demonstrate virus in nasal or oral swabs, indicating that the virus is not secreted and/or excreted by infected animals, nor could trans-placental infection in these animals be demonstrated (Planzer *et al.* 2011). Interestingly, a recent study has reported long term persistence of infectious BTV-25 in goats for up to 19 months; however the route of virus transmission in this study was not established (Waldvogel *et al.* 1987).

Bluetongue virus serotype 26 was discovered in sheep and goats in Kuwait in 2010 (Maan *et al.* 2011). Similar to BTV-25, experimental infection of goats seems to indicate that goats are the natural reservoir of the virus, as high viraemia but an absence of clinical signs is associated with infection in this host species (Batten *et al.* 2011, Batten *et al.* 2013). The finding that it is not possible to infect *C. sonorensis* midges with BTV-26 using oral feeding on virus spiked blood-meals, has led to research into possible alternative transmission routes. In this regard, a recent study has demonstrated that BTV-26 transmission can occur between goats through direct contact transmission (Batten *et al.* 2011).

Future directions

Despite significant advances that have been made in the understanding of BTV-vector-host interaction in recent years, several questions still remain unanswered. As pertaining to the work discussed in this article, the cellular pathways involved in the induction of innate immunity, as well as the function of NS4 in the BTV replication cycle, have not completely been delineated. The identities of the specific molecular determinants and influence of quasispecies diversity in determining BTV phenotype is still unclear, whereas the role of viral associated vector infectivity factors in influencing BTV transmission and BT disease severity, are only now being investigated. The role of BTV evolutionary processes, including reassortment and/or recombination in influencing BTV phenotype over short evolutionary time periods is unknown; whereas many key questions regarding the pathogenesis of BT remain to be addressed. The precise pathological mechanisms involved in BTV trans-placental transmission and seminal shedding for example requires further study. From the perspective of vaccinology, several new vaccine approaches have been developed. However, additional research is required to evaluate their long-term safety, stability, and efficacy. Finally, the mechanisms involved in BTV overwintering, and in particularly BTV-8 overwintering in Northern Europe remains unclear, as well as possible transmission routes of BTV-25 and BTV-26.

References

Barros S.C., Ramos F., Luis T. M., Vaz A., Duarte M., Henriques M., Cruz B. & Fevereiro M. 2007. Molecular epidemiology of bluetongue virus in Portugal during 2004-2006 outbreak. *Vet Microbiol*, **124**, 25-34.

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., Henstock M.R., Bin-Tarif A., Steedman H.M., Waddington S., Edwards L. & Oura C.A. 2011. Bluetongue virus serotype 26: infection kinetics and pathogenesis in Dorset Poll sheep. *Vet Microbiol*, **157**, 119-24.

Batten C.A., Henstock M. R., Steedman H. M., Waddington S., Edwards L. & Oura C. A. 2013. Bluetongue virus serotype 26: infection kinetics, pathogenesis and possible contact transmission in goats. *Vet Microbiol*, **162**, 62-67.

Belhouchet M., Mohd J.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.

Bouwknegt C., van Rijn P.A., Schipper J.J., Holzel D., Boonstra J., Nijhof A.M., van Rooij E.M. & Jongejan F. 2010. Potential role of ticks as vectors of bluetongue virus. *Exp Appl Acarol*, **52**, 183-192.

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.

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

Campbell C.L., Vandyke K.A., Letchworth G.J., Drolet B.S., Hanekamp T. & Wilson W.C. 2005. Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). *Insect Mol Biol*, **14**, 121-136.

Caporale M., Di Gialleonardo 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.

Chaignat V., Worwa G., Scherrer N., Hilbe M., Ehrensperger F., Batten C., Cortyen M., Hofmann M. & Thuer B. 2009. Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. *Vet Microbiol*, **138**, 11-19.

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.

Clausen P.H., Stephan A., Bartsch S., Jandowsky A., Hoffmann-Kohler P., Schein E., Mehlitz D. & Bauer B. 2009. Seasonal dynamics of biting midges (Diptera: Ceratopogonidae, *Culicoides* spp.) on dairy farms of Central Germany during the 2007/2008 epidemic of bluetongue. *Parasitol Res*, **105**, 381-386.

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., Venter E.H. & Stokstad M. 2014. A review of experimental infections with bluetongue virus in the mammalian host. *Virus Res*, **182**, 21-34.

Dal Pozzo F., Martinelle L., Thys C., Sarradin P., De Leeuw I., Van Campe W., De Clercq K., Thiry E. & Saegerman C. 2013. Experimental co-infections of calves with bluetongue virus serotypes 1 and 8. *Vet Microbiol*, **165**, 167-172.

Darpel K.E., Langner K.F., Nimtz M., Anthony S.J., Brownlie J., Takamatsu H.H., Mellor P.S. & Mertens P.P. 2011. Saliva proteins of vector *Culicoides* modify structure and infectivity of bluetongue virus particles. *PLoS One*, **6**, e17545.

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., De Leeuw I., Verheyden B., Vandemeulebroucke E., Vanbinst T., Herr C., Meroc E., Bertels G., Steurbaut N., Miry C., De Bleecker K., Maquet G., Bughin J., Saulmont M., Lebrun M., Sustronck B., De Deken R., Hooyberghs J., Houdart P., Raemaekers M., Mintiens K., Kerkhofs P., Goris N. & Vandenbussche F. 2008. Transplacental infection and apparently immunotolerance induced by a wild-type bluetongue virus serotype 8 natural infection. *Transbound Emerg Dis*, **55**, 352-359.

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-Thie 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.

Di Gialleonardo L., Migliaccio P., Teodori L. & Savini G. 2011. The length of BTV-8 viraemia in cattle according to infection doses and diagnostic techniques. *Res Vet Sci*, **91**, 316-320.

Drew C.P., Gardner I.A., Mayo C.E., Matsuo E., Roy P. & MacLachlan N.J. 2010. Bluetongue virus infection

alters the impedance of monolayers of bovine endothelial cells as a result of cell death. *Vet Immunol Immunopathol*, **136**, 108-115.

Eschbaumer M., Wackerlin R., Rudolf M., Keller M., Konig P., Zemke J., Hoffmann B. & Beer M. 2010. Infectious blood or culture-grown virus: a comparison of bluetongue virus challenge models. *Vet Microbiol*, **146**, 150-154.

Feenstra F., van Gennip R.G., Maris-Veldhuis M., Verheij E. & van Rijn P.A. 2014. Bluetongue virus without NS3/NS3a expression is not virulent and protects against virulent BTV challenge. *J Gen Virol*, **95** (Pt 9), 2019-2029. doi: 10.1099/vir.0.065615-0.

Gold S., Monaghan P., Mertens P. & Jackson T. 2010. A clathrin independent macropinocytosis-like entry mechanism used by bluetongue virus-1 during infection of BHK cells. *PLoS One*, **5**, e11360.

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.

Huismans H. & Howell P.G. 1973. Molecular hybridization studies on the relationships between different serotypes of bluetongue virus and on the difference between the virulent and attenuated strains of the same serotype. *Onderstepoort J Vet Res*, **40**, 93-103.

Huismans H., van Staden V., Fick W.C., van Niekerk M. & Meiring T.L. 2004. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. *Vet Ital*, **40**, 417-425.

Kirkland P. & Hawkes R.A. 2004. A comparison of laboratory and 'wild' strains of bluetongue virus - is there any difference and does it matter? *Vet Ital*, **40**, 448-455.

Leemans J., Raes M., Vanbinst T., De Clerck K., Saegerman C. & Kirschvink N. 2011. Viral RNA load in semen from bluetongue serotype 8-infected rams: relationship with sperm quality. *Vet J*, **192**, 304-310.

Lehiy C.J. & Drolet B.S. 2014. The salivary secretome of the biting midge, *Culicoides sonorensis*. *Peer J*, **2**, e426.

Maan S., Maan N.S., Nomikou K., Batten C., Antony F., Belaganahalli M.N., Samy A.M., Reda A.A., Al-Rashid S.A., El Batel M., Oura C.A. & Mertens P.P. 2011. Novel bluetongue virus serotype from Kuwait. *Emerg Infect Dis*, **17**, 886-889.

MacLachlan N.J. 2011. Bluetongue: history, global epidemiology, and pathogenesis. *Prev Vet Med*, **102**, 107-111.

Matsuo E., Celma C.C., Boyce M., Viarouge C., Sailleau C., Dubois E., Breard E., Thiery R., Zientara S. & Roy P. 2011. Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. *J Virol*, **85**, 10213-10221.

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.

Mohl B.P. & Roy P. 2014. Bluetongue virus capsid assembly and maturation. *Viruses*, **6**, 3250-3270.

Mortola E., Noad R. & Roy P. 2004. Bluetongue virus outer capsid proteins are sufficient to trigger apoptosis in mammalian cells. *J Virol*, **78**, 2875-2883.

Muller U., Kemmerling K., Straet D., Janowitz U. & Sauerwein H. 2010. Effects of bluetongue virus infection on sperm quality in bulls: a preliminary report. *Vet J*, **186**, 402-403.

Noad R. & Roy P. 2009. Bluetongue vaccines. *Vaccine*, Suppl **4**, D86-89. doi: 10.1016/j.vaccine.2009.08.037.

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 Suppl*, **14**, 259-279.

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.

Planzer J., Kaufmann C., Worwa G., Gavier-Widen D., Hofmann M.A., Chaignat V. & Thur B. 2011. *In vivo* and *in vitro* propagation and transmission of Toggenburg orbivirus. *Res Vet Sci*, **91**, e163-e168.

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.

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.

Sanchez-Cordon P.J., Pedrera M., Risalde M.A., Molina V., Rodriguez-Sanchez B., Nunez A., Sanchez-Vizcaino J. M. & Gomez-Villamandos J.C. 2012. Potential role of proinflammatory cytokines in the pathogenetic mechanisms of vascular lesions in goats naturally infected with Bluetongue virus serotype 1. *Transbound Emerg Dis*, **60**, 252-262.

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.

Savini G., Annas C., 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., Lorusso A., Paladini C., Migliaccio P., Di Gennaro A., Di Provvido A., Scacchia M. & Monaco F. 2014. Bluetongue serotype 2 and 9 modified live vaccine viruses as causative agents of abortion in livestock: a retrospective analysis in Italy. *Transbound Emerg Dis*, **61**, 69-74.

Shaw A.E., Veronesi E., Maurin G., Ftaich N., Guiguen F., Rixon F., Ratinier M., Mertens P., Carpenter S., Palmarini M., Terzian C. & Arnaud F. 2012. *Drosophila melanogaster* as a model organism for bluetongue virus replication and tropism. *J Virol*, **86**, 9015-9024.

Stewart M.E. & Roy P. 2010. Role of cellular caspases, nuclear factor-kappa B and interferon regulatory factors in Bluetongue virus infection and cell fate. *Virol J*, **7**, 362.

Umeshappa C.S., Singh K.P., Channappanavar R., Sharma K., Nanjundappa R.H., Saxena M., Singh R. & Sharma A. K. 2011. A comparison of intradermal and intravenous inoculation of bluetongue virus serotype 23 in sheep for clinico-pathology, and viral and immune responses. *Vet Immunol Immunopathol*, **141**, 230-238.

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.

Vogtlin A., Hofmann M.A., Nenniger C., Renzullo S., Steinrigl A., Loitsch A., Schwermer H., Kaufmann C. & Thur B. 2013. Long-term infection of goats with bluetongue virus serotype 25. *Vet Microbiol*, **166**, 165-173.

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. Association of virulent and avirulent strains of bluetongue virus serotype 11 with premature births of late-term bovine fetuses. *J Comp Pathol*, **106**, 333-340.

White D.M., Wilson W.C., Blair C.D. & Beaty B.J. 2005. Studies on overwintering of bluetongue viruses in insects. *J Gen Virol*, **86**, 453-462.