A review of experimental infections with bluetongue virus in the mammalian host

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Abstract

Experimental infection studies with bluetongue virus (BTV) in the mammalian host have a history that stretches back to the late 18th century. Studies in a wide range of ruminant and camelid species as well as mice have been instrumental in understanding BTV transmission, bluetongue (BT) pathogenicity/pathogenesis, viral virulence, the induced immune response, as well as reproductive failures associated with BTV infection. These studies have in many cases been complemented by *in vitro* studies with BTV in different cell types in tissue culture. Together these studies have formed the basis for the understanding of BTV-host interaction and have contributed to the design of successful control strategies, including the development of effective vaccines. This review describes some of the fundamental and contemporary infection studies that have been conducted with BTV in the mammalian host and provides an overview of the principal animal welfare issues that should be considered when designing experimental infection studies with BTV in *in vivo* infection models. Examples are provided from the authors' own laboratory where the three Rs (replacement, reduction and refinement) have been implemented in the design of experimental infection

studies with BTV in mice and goats. The use of the ARRIVE guidelines for the reporting of data from animal infection studies is emphasised.

Keywords

Bluetongue, bluetongue virus, experimental infection, pathogenicity, pathology, virulence, immunity, animal welfare

Contents

1. Introduction and brief historical overview

Experimental infections with bluetongue virus (BTV) have been an important research tool ever since the disease was first documented in the late 1800s. One of the earliest reports

published by the Cattle and Sheep Diseases Commission in South Africa in 1876 described the disease as it was experienced in imported susceptible European breeds of sheep (cited by Henning, 1949). Studies of a more systematic nature were documented early in the 20th century that included inoculation of sheep with infected blood. In one study the infectivity of a single drop of blood from a BTV infected sheep was reported to be sufficient to establish infection in a susceptible recipient sheep (Spreul, 1902; Spreull, 1905).

Experimental infections that focussed on establishing the vector-borne nature of bluetongue (BT) were first documented in South Africa in 1944 following classical experiments in which BTV was transmitted to susceptible sheep by inoculation of homogenized pools of wild-caught midges of the species *Culicoides imicola* (Du Toit, 1944). These experiments were preceded and made possible through the development of a light trap for catching of large numbers of the insects. Midges of the same species, which were subsequently fed on a sheep infected with BTV, were shown to be able to transmit the virus through bites to other sheep (Du Toit, 1944). This finding was confirmed under controlled conditions in the USA, but with *C. variipennis* (Foster *et al.*, 1963). Twenty and fort years respectively after the experiments by Du Toit, the disease was transmitted experimentally with the sheep ked, *Melophagus ovinus* (Luedke *et al.*, 1965) and a soft tick *Ornithodoros coriaceus* (Stott *et al.*, 1985b), however a biological cycle could not be demonstrated in these arthropods.

Experimental infections with BTV have been accomplished by means of a variety of inoculation routes or combination of routes (Umeshappa *et al.*, 2011) as well as with ruminant and cell culture passaged virus (Eschbaumer *et al.*, 2010). For these studies, the use of well-defined virus stocks in order to study differences in the same species/breed as well as to study differences in the same species by different viruses was essential to make meaningful comparisons, due to potential phenotypic differences that exist between virus strains (e.g. virulence). Through experimental infections it became clear that the outcome of infection varied between different species and breeds as well as among individuals of the same species. Experimental infections of susceptible sheep with field strains of BTV, including strains that caused severe disease in sheep, often result only in mild clinical signs (Verwoerd & Erasmus, 2004). Similarly, the clinical signs observed after natural infection in indigenous breeds of sheep were less severe than those observed in exotic or improved breeds. Clinical signs have similarly been found to be milder in cattle and goats than in sheep, although severe disease has occasionally been observed in these species after exposure to certain strains and/or under

specific environmental conditions (Dal Pozzo *et al*., 2009; Elbers *et al*., 2008; Guyot *et al*., 2008; Zanella *et al*., 2012).

2. Experimental infections to study the pathogenicity and virulence of BTV in different hosts

Experimental reproduction of bluetongue (BT) may serve the purpose of developing a reliable model to facilitate pathogenic studies of the disease and several examples of such models are available in the literature. Two contemporary examples in sheep and cattle are described by MacLachlan *et al.,* (2008) and Dal Pozzo *et al*., (2009). MacLachlan *et al*., (2008) inoculated sheep with a highly virulent South African BTV-4 strain. Sheep inoculated intravenously with infected sheep blood, developed fulminant BT, characterized by high fever, serous nasal discharge, respiratory distress, facial edema, congestion, haemorrhage and ulceration of the oral mucous membranes and coronitis. Gross lesions included ulceration of the mucosal lining of the oral cavity and forestomachs, haemorrhage in the wall of the pulmonary artery, focally extensive necrosis of skeletal muscle, and in some of the animals, cyanosis of the tongue. The authors further describe particularly severe pulmonary oedema, oedema of the subcutaneous tissues and fascial planes of the head and neck, as well as pleural and pericardial effusion of varying severity (MacLachlan *et al.*, 2008). A similar effort to develop a model for the study of the pathogenesis of BTV-8 in cattle motivated researchers in Europe to infect two calves both intravenously and subcutaneously with cell culture passaged virus. Calves developed clinical signs including fever, ocular discharge, conjunctivitis, oral mucosal congestion, ulcers and necrotic lesions on the lips and tongue, submandibular oedema, coronitis and oedema of the coronet and pastern region (Dal Pozzo *et al.*, 2009).

To reduce the dependence on cattle and sheep, earlier studies were conducted to evaluate mice as hosts for virus isolation and indicators of viral attenuation. It was found that suckling mice are highly susceptible to infection to BTV, in particular when inoculated via the intra-cranial route. In a seminal paper Narayan and Johnson, (1972) described the pathogenicity of BTV infection in mice of varying ages. The study found that BTV selectively targets immature cells of the sub-ventricular zone of the forebrain of suckling mice from where it migrates along cellular migratory pathways during post-natal maturation to the olfactory bulbs, caudate/putamen, hippocampus and areas of the cerebral cortex. In mice, infection of brain tissue caused necrotizing encephalitis and the development of cavitating lesions, similar

to what is observed when ovine and/or bovine foetuses are infected with BTV during early gestation. The study also described the exquisite age susceptibility of mice to infection with BTV. The authors found that the susceptibility of mice to BTV infection decreases rapidly with age, with two-week-old mice being largely refractory to infection.

In order to overcome the age dependency of BTV infection in mice, the establishment of a new laboratory model using alpha/beta interferon receptor-deficient ($IFNAR^{-/-}$) mice for the study of orbivirus infections in general and specifically BTV was recently reported by Calvo-Pinilla *et al*., (2009). Alpha/beta interferon receptor-deficient mice are highly susceptible to BTV at any age using various inoculation routes and demonstrate similar BTV tissue tropism and gross lesions as the ruminant host (Calvo-Pinilla *et al.*, 2010). This animal model greatly facilitates preliminary studies on immune responses to BTV vaccination strategies (Calvo-Pinilla *et al.*, 2010). Experimental inoculation of $IFNAR^{-/-}$ mice for example using a BTV-4 inactivated vaccine has been shown to lead to the induction of neutralising antibodies against the virus that confer complete protection against a lethal BTV-4 challenge. In more recent studies $IFNAR^{-/-}$mice have been used to evaluate the immune response induced by several experimental recombinant vaccine candidates. (Calvo-Pinilla *et al.*, 2012; Franceschi *et al.*, 2011; Jabbar *et al.*, 2013; Ma *et al.*, 2012). It should be noted that the use of $IFNAR^{-/-}$ mice in BTV challenge and/or vaccine efficacy studies should be interpreted with caution, due to the lack of an intact interferon response. Viral replication and spread in $IFNAR^{-/-}$ mice may be promoted, especially during early infection. In cattle for example high levels of interferon in the peripheral circulation precedes increases in BTV titres, suggesting that the interferon response may play a role in initial antiviral response against the virus early during infection (MacLachlan and Thompson, 1985). Vaccine efficacy and immunity studies should therefore always be repeated in the native ruminant host, in order to confirm findings in $IFNAR^{-/-}$ mice.

Virulence characteristics vary between BTV strains independent of serotype and are reflected in the substantial genetic diversity that occurs amongst BTV strains in the fields (Bonneau & MacLachlan, 2004; Caporale *et al.*, 2011). Currently the determinants of BTV virulence are not well understood. Huisman *et al*., (2004) suggested that the efficiency of viral attachment and penetration, the overall rate of viral replication, the efficiency/mechanism of virus release, together with associated membrane damage, cell death and virus spread may be involved in altered virulence characteristics of BTV strains *in vivo* (Huismans *et al.*, 2004). For BTV this has focused attention primarily on viral proteins associated with cell attachment

(VP2), penetration (VP5) and release (NS3/NS3a) (Bernard *et al.*, 1996; Caporale *et al.*, 2011; Carr *et al.*, 1994; Gould & Eaton, 1990; Huismans *et al.*, 2004; Huismans & Howell, 1973; Owens *et al.*, 2004). Other studies have implicated variation in VP1, VP2, VP5 and NS2 as being associated with changes in BTV virulence in experimentally infected mice (Caporale *et al.*, 2011; Carr *et al.*, 1994; Waldvogel *et al.*, 1986).

Differences in virulence in the published literature are in general described in terms of specific viral isolates or local circulating strains. The virulence of BTV strains has been determined by means of comparative clinical responses by inoculating sheep with different strains of the virus under the same experimental conditions. In this respect, an Australian BTV-20 strain was compared to an American BTV-17 strain which had shown cross-reactions in a serum-neutralisation test (Groocock *et al.*, 1982). Although no mortalities occurred, there were clear differences in terms of the development of fever and hyperaemia of the naso-labial area and oral mucosa, as well as the time of first detection and duration of viraemia. In another study, an assessment of the virulence of a BTV strain inoculated into 3 different breeds of susceptible British sheep under stressful conditions was conducted by means of subcutaneous inoculation of a South African BTV-3 strain. Parameters used to evaluate the virulence of the strain included the development of clinical signs, mortality and changes in serum enzyme levels (Jeggo *et al.*, 1987). Other research groups likewise studied the virulence of BTV in specific host species. Experimental infection of West African dwarf sheep with a Nigerian strain of BTV-7 did not yield any clinical signs when animals were inoculated subcutaneously (Tomori, 1980). However, viraemia and development of complement fixing and neutralising antibodies were detected in non-immune sheep. In Greece, the duration of viraemia and serological response were studied in two local sheep breeds and two local breeds of goats experimentally infected with a strain of BTV-4. Animals were injected intradermally at four different sites (ears and inner thighs). Significant differences in detectable viraemias between sheep and goats included in the study were not recorded but extended viraemias averaging up to 41 days were documented (Koumbati *et al.*, 1999). Experimental infections with European strains of BTV-1 and BTV-8 led to observations that suggested the existence of a direct link between the pathogenicity of BTV serotypes, the severity of vascular lesions and the serum concentrations of acute phase proteins (Sanchez-Cordon *et al.*, 2013). The authors injected sheep subcutaneously in the axillar region and showed that BTV-1 was more virulent than BTV-8 by virtue of the fact that

the clinical course of the disease was longer, with a significant increase in clinical signs and more severe gross lesions than BTV-8 infected sheep.

The discovery of new bluetongue serotypes requires experimental infections to determine the infection kinetics, pathogenicity and virulence of the viruses. Detection of a novel BTV in goats in Switzerland in 2008 termed Toggenburg orbivirus (TOV) necessitated experimental infection of goats and sheep, using blood from TOV-positive field cases, as efforts to propagate the virus in laboratory host systems have been unsuccessful. Although goats did not show any clinical or pathological signs, mild signs were observed in sheep including the haemorrhages in the wall of the pulmonary artery that has historically strongly been associated with BTV infection in sheep (Chaignat *et al.*, 2009; Planzer *et al.*, 2009). More recently experimental infection studies have been conducted with a novel BTV-26 that was isolated from sheep and goats in Kuwait in 2010. In these studies, a strain of BTV-26 that was isolated from an infected sheep in hen eggs and/or baby hamster kidney cells was inoculated subcutaneously into 6 Dorset Poll sheep (Batten *et al*., 2012) and 5 goats (Batten *et al*., 2013). Although goats did not show any clinical or pathological signs, mild clinical signs were observed in sheep including conjunctivitis, reddening of the mouth and mucosal membranes, slight oedema of the face and nasal discharge. Gross lesions included haemorrhages in the spleen, interstitial oedema of the lungs and hydro-pericardium. Goats in particular demonstrated high levels of RNA in their blood that together with the absence of clinical signs/pathological lesions suggests that goats are the natural host for the virus.

Following reports of deaths and abortions among pregnant bitches that were vaccinated with a BTV-11 contaminated canine coronavirus vaccine (Akita *et al.*, 1994), Brown and co-workers (1996) studied the effect of inoculation of BTV-11 in pregnant and non-pregnant dogs. The non-pregnant dogs did not develop clinical signs, but 3 of the 4 pregnant bitches aborted. The predominant pathological lesion observed was severe pulmonary oedema (Brown *et al*., 1996).

3. Experimental infections in wild ruminants and camelids

As a result of the economic importance of BT in sheep, and especially the occurrence of major epidemics amongst this host species, BT has traditionally been associated with sheep. However, BT affects a wide range of species. The susceptibility of wild ruminants was first established in South Africa in 1933 by experimental infection of blesbuck

(*Damaliscus albifrons*) (Neitz, 1933). This antelope species developed a sub-clinical infection with a sufficiently high viraemia to infect sheep when experimentally injected with infected blood. It is now generally accepted that all ruminant species are susceptible to infection with BTV. Although African antelope do not develop clinical disease, white-tailed deer (*Odocoileus virginianus*), pronghorn (*Antilocapra americana*) and desert bighorn sheep (*Ovis canadensis*) of the North American continent may develop severe clinical disease (Hoff, 1976).

The white-tailed deer (WTD) has been studied the most closely in terms of the effect of BTV on wild ruminants. Experimental infections in WTD in the USA in 1968 were conducted with either blood or filtered/unfiltered spleen as inocula and delivered by both intravenous and intramuscular routes (Vosdingh *et al.*, 1968). In the latter study, 9 deer were experimentally infected, 7 of which developed fatal bluetongue. Similar experimental infections with BTV in black-tailed deer were done to determine if the virus could be responsible for haemorrhagic disease in that species but with negative results (Work *et al.*, 1992). The authors used several routes of inoculation but only observed fever up to 3 dpi (6 days in the case of one animal). Experimentally induced BTV infection in WTD to study ultra-structural changes emphasised striking changes in the endothelial lining of the microvasculature by post-inoculation day 4. Endothelial cell degeneration and necrosis, which resulted in denudation of the endothelial lining, and endothelial cell hypertrophy were observed, as were thrombosis, haemorrhage, and vessel rupture that developed subsequent to endothelial damage. It was concluded that vascular damage coupled with the development of disseminated intravascular coagulation is responsible for the haemorrhagic diathesis, which is characteristic of BTV infection in WTD (Howerth & Tyler, 1988). Howerth and co-workers also inoculated ten yearling WTD with BTV-17 and found widespread haemorrhage, which ranged from petechiae to massive haematoma formation. Haemorrhage was accompanied by abnormal values in a wide range of clotting factors (Howerth *et al.*, 1988). Experimental infections of WTD with BTV were recently repeated in the USA to study the risk that the newly emerged European strain of BTV-8 may have for North American WTD (Drolet *et al.*, 2013). Results from the study led the authors to conclude that North American WTD are highly susceptible to BTV-8 and would act as clinical disease sentinels and amplifying hosts during an outbreak. Infection of European red deer has been also been conducted in Europe following the 2006-2008 outbreak of BTV-8. Bluetongue virus RNA was detected in European red deer blood for long periods, comparable to those of domestic ruminants, after experimental infection with BTV-1 and

BTV-8. Bluetongue virus RNA was detected up to the end of the study (98-112 dpi). The results prompted the authors to conclude that red deer can be infected with BTV and maintain viral RNA for long periods, remaining essentially asymptomatic (Lopez-Olvera *et al.*, 2010).

The susceptibility of other wild ungulates/camelids has been explored in several studies. In one study six yearling American bison (*Bison bison*) bulls were inoculated intradermally and subcutaneously with 2 x 10^5 plaque forming units of BTV-11. Although BT viraemia was detected in all six inoculated bison, pooled blood samples collected at 28, 56, 84 and 112 dpi from the six infected bison were not infectious for sheep. No clinical signs or lesions attributable to BT were observed in the infected bison or control animals (Tessaro & Clavijo, 2001).

The potential role of camels as reservoirs for BTV received prominence when BT emerged in Morocco in 2004 where it was always regarded as an exotic disease. Bluetongue virus-derived clinical disease had never previously been observed in camels. Experimental infection of 3 camels with a Moroccan BTV-1 isolate via the subcutaneous, intramuscular and intravenous routes did not yield any clinical signs. However, virus was isolated from the blood of all three animals, leading to the conclusion by the authors that camels may act as a reservoir for BTV and play a role in its transmission (Batten *et al.*, 2011). However, the lowest threshold cycle (Ct) value with a real-time RT-PCR in the camels in that study was 31.88 (as opposed to Ct values as low as 20 documented in sheep in other studies) which suggested that BTV replication may not be as efficient in camels as in sheep.

Historically, South American camelids (SAC) were considered to be resistant to BTV-induced disease. Experimental BTV-10 infections have only been conducted in two llamas (*Lama glama*) prior to 2013 to evaluate a competitive ELISA (cELISA), but no clinical signs were observed (Afshar *et al*., 1995). Fatalities related to BTV in captive SAC following the emergence of BT-8 throughout Europe, raised questions about the possible role of SAC in BTV epidemiology. In order to answer these questions, 3 alpacas (*Vicugna pacos*) and 3 llamas (*Lama glama*) were experimentally infected subcutaneously with a German BTV-8 isolate. The animals displayed only very mild clinical signs. Virus isolation was only possible from blood samples of two alpacas by inoculation of IFNAR$^{(-/-)}$ mice. In contrast to the conclusion drawn about the possible role of Old World camelids in the transmission of BTV,

the experimental infections in SAC pointed to a negligible role for these animals in BTV epidemiology (Schulz *et al.*, 2012).

4. Experimental infections to study the pathology of bluetongue

The majority of experimental infections with BTV are based on the infection of experimental animals through needle injection. It has quite correctly been pointed out that this is not a natural route of infection and that the extent to which it mimics the natural transmission of the virus by *Culicoides* midges is not known (Darpel *et al.*, 2012). It is likewise uncertain whether the early development of clinical signs in experimental animals (and by implication pathological lesions) may have been partly due to artificial infection routes. It was recently reported that the intradermal route of infection in BTV-23 inoculated sheep, in contrast to the intravenous route, led to an earlier onset of clinical signs, increased antibody titres in the blood and more severe clinical signs/pathological lesions in infected animals. The results from this study led the authors to conclude that the intradermal route may be useful in setting up experimental infections for challenge and/or pathogenesis studies. Subcutaneous inoculation also appears to simulate the natural route of infection more closely than the intravenous inoculation route, in respect to the dissemination of the virus from the skin to secondary target organs as has been observed following natural infection (Umeshappa *et al.*, 2011)

Irrespective of the route of infection, experimental BTV infections have made it possible to perform gross pathology and ultra-structural studies (Mahrt & Osburn, 1986). An infection protocol applied in sheep in 2008 (Worwa *et al.*, 2008) was applied again in 2010 and entailed intradermal and subcutaneous inoculation of 3 sheep with 2.1 mL of 1:2 diluted cattle blood containing the northern European field strain of BTV-8 from a 2007 outbreak in Germany. The blood sample was tested for viral RNA levels by qRT-PCR and yielded a cycle threshold value (Ct value) of 24.9. Subsequently infectious blood with a Ct value of 25.0 was obtained from the 3 sheep and used to inoculate twenty four sheep representing 4 different Swiss breeds and one British breed. The authors used a scoring system to show that clinical manifestation and the severity of pathological lesions were significantly related (Worwa *et al.*, 2010). In another study, in an effort to pre-empt the clinical and pathological effects of the newly emerged European BTV-8 strain on British sheep and cattle prior to its appearance in that country, experimental studies were conducted on British poll Dorset sheep

and Holstein-Friesian cattle (Darpel *et al.*, 2007). The sheep were inoculated with 1 ml of strain NET2006/01 subcutaneously in the neck, and with 0·5 ml of strain BTV-8-E1 intradermally into the inner left leg. The calves were inoculated with 2 ml of strain NET2006/01 subcutaneously and with 0·5 ml BTV-8-E1 intradermally along the back/flank. The authors pointed out that despite the use of identical doses of virus, the severity of the clinical signs varied significantly between individuals, with severe clinical signs in two of the sheep (which would likely have led to death under field conditions) but relatively mild signs in two others. It was further emphasised that although the clinical signs in calves were mild, the post mortem lesions were more pronounced. The high degree of variation, combined with the known interbreed variation in susceptibility to BTV have also been documented by other researchers (MacLachlan, 1994; Parsonson *et al.*, 1987; Richards *et al.*, 1988). It is noteworthy that the outcome of experimental infection is also dependent on the age of infected animals. For example young calves of 3-4 weeks show delayed seroconversion, whereas cattle older than six 6 months show 'normal' seroconversion (van Rijn *et al*., 2012).

5. Experimental infections to study the immune response to BTV

Both humoral and cell-mediated immune responses are activated by animals infected with BTV (MacLachlan, 1994; MacLachlan & Thompson, 1985). The immune response following exposure to BTV has been investigated with the aid of experimental infections in mice and ruminants, mainly with the aim of testing the efficacy of vaccines or improving existing vaccines.

(i) Serotype-specific protection and heterotypic immunity

It has long been known that sheep that have recovered from BT demonstrate some degree of immunity to reinfection (Spreul, 1902; Spreull, 1905). Both Spreull and Theiler (1906) proposed approaches to immunization of sheep (Theiler, 1906), but the first polyvalent vaccine was developed by Alexander in 1940 (Mason *et al.*, 1940). The cross-protection between different BTV serotypes in sheep were first extensively studied in the late 1940s when Neitz (1948) showed that immunized sheep remained susceptible to infection with heterologous BTV serotypes, i.e. infection by one serotype provided only partial to no protection against other BTV serotypes (Neitz, 1948). Conversely, BTV-inoculated animals can also develop serotype-specific (neutralising) antibodies to serotypes to which they have

not previously been exposed (Dungu *et al*., 2004; Erasmus, 1990; Maan *et al*., 2007). Jeggo and co-workers (1983) showed that sheep previously exposed to BTV-3 and BTV-4 were resistant to BTV-6, and also developed neutralising antibodies to other serotypes. However, these authors failed to raise heterotypic neutralising antibodies when only two of three viruses replicated after simultaneous inoculation of sheep with three different serotypes (Jeggo *et al.*, 1986). These results raised concerns regarding the use of polyvalent live attenuated vaccines (MLVs), as insufficient humoral immunity may develop against some serotypes (Jeggo & Wardley, 1985). In a recent joint study by the University of Pretoria and Onderstepoort Biological Products, a BTV-4 MLV strain could protect against BTV-9 and BTV-11 challenges but not against BTV-1 and BTV-10 (Zulu, 2014).

It was demonstrated that differences in virulence between isolates play a role in the immune response (Neitz, 1948). Since each BTV serotype reacts differently and the immunogenic potential differs from serotype to serotype (Howell, 1969), Modumo & Venter (2012) infected sheep with BTV-2 and BTV-8 vaccine strains with titres of 10^2, 10^3 and 10^4 plaque forming units (PFU)/mL. Experimental infection with these different viral titres and strains were done to evaluate the minimum dose at which the vaccine strains were still protective and safe (Modumo & Venter, 2012). Sheep (12 per serotype, 4 per titre) were infected, monitored and challenged with the homologous serotype. Considering the protection index in sheep obtained in this study, it is recommended that for a BTV-2 vaccine, sheep should be vaccinated with a titre of 10^3 PFU/mL and a titre of 10^2 PFU/mL for a BTV -8 vaccine. Therefore during production and release of polyvalent MLVs, titres of specific serotypes should be considered, rather than the average of all titres in a batch, as is currently practiced (Modumo & Venter, 2012). More work remains to be undertaken to quantify the duration and level of viraemia post vaccination and post challenge, especially when low titres of MLV are administered. Low titre vaccines should also be tested when included in a polyvalent format, as opposed to monovalent vaccines in particular with respect to lifelong immunity.

It should be noted that studies to evaluate the protective dose and/or vaccine safety in endemic regions using local sheep breeds may not necessarily be indicative of the safety of vaccine strains in sheep in non-endemic regions. Concerns have been raised about the level and duration of viraemia that may sometimes occur after vaccination with MLVs, especially in sheep (Dungu *et al.*, 2004). Further experimental infection studies have indicated that MLVs may cause severe clinical signs (Dungu *et al.*, 2004; Veronesi *et al*., 2005; Veronesi *et*

al., 2009). Veronesi and co-workers (2005) demonstrated that viraemias in BTV-2 and BTV-9 vaccinated Dorset Poll sheep were in some cases sufficiently high (> 3 ^{10}log $TCID_{50}$/ml) and of a long enough duration (up to 17 days) to promote the transmission of the strains in the field. In a second study following vaccination of Dorset Poll sheep with BTV-4 and BTV-16 MLV titres varying from 3.5 to 6.83 ^{10}log $TCID_{50}$/ml were recorded in infected sheep and infectious viraemias lasted from 9 to 23 days. Further vaccination of sheep with these strains, resulted in severe clinical signs including pyrexia, respiratory distress, cyanosis of the mucous membranes and oedema of the face and lips (Veronesi *et al*., 2009). Conversely vaccination of sheep in South Africa does not result in BT clinical signs, apart from transient pyrexia (Dungu *et al*., 2004). Due to the potential of MLV strains to cause disease in European sheep breeds, the use of MLVs in countries of northern Europe have been prohibited (Zientara, 2013).

The differences in the genetic susceptibility of sheep to BTV infection was confirmed by a study on the immune response by different sheep breeds and suggested the difference in disease expression may also in part be due to the genetic differences in humoral and cellular immune responses in different sheep breeds (Stott *et al.*, 1985a). Cellular immunity may also play a role in heterotypic immunity and cross-reactive lysis of cytotoxic T-lymphocytes was reported by Jeggo & Wardley (1982a; 1985). These authors (1982a) reported that BTV-4 immune mice inoculated with BTV-16 produced cytotoxic T-lymphocytes which lysed BTV-10 infected target cells (Jeggo & Wardley, 1982a; Jeggo & Wardley, 1985).

(ii) Humoral immunity /neutralising antibodies

Serotype-specific neutralising antibodies against the VP2 outer capsid protein confers protection against homologous strain reinfection in sheep (Roy *et al.*, 1992; Schwartz-Cornil *et al.*, 2008) and neutralising antibodies can also be induced, to a lesser degree, by the VP5 protein (Lobato *et al.*, 1997; Roy *et al.*, 1992). The sera of infected ruminants also contain serogroup-reactive antibodies induced by the more conserved virus proteins e.g. VP7, as well as antibodies against other structural and non-structural proteins. Production of antibodies against proteins other than VP2/VP5 have however, not been correlated to a protective humoral response (Huismans & Erasmus, 1981; MacLachlan *et al.*, 1987).

Individually expressed BTV proteins and the use of BTV-like particles (VLP) and core-like particles (CLP) as vaccines in clinical trials in BTV-susceptible cattle and sheep have been studied extensively (Celma *et al.*, 2013; Perez de Diego *et al.*, 2011; Roy, 2003; Stewart *et al.*, 2012). Virus-like particles are highly immunogenic structural mimics of virus particles, and only contain a subset of the proteins present in a natural infection. Virus-like particles therefore offer the potential for the development of DIVA ("Differentiating Infected from Vaccinated Animals") compatible BT vaccines. Diego and co-workers (2011) vaccinated merino sheep with either monovalent BTV-1 VLPs or a bivalent mixture of BTV-1 and BTV-4 VLPs, and challenged the animals with virulent BTV-1 or BTV-4. Animals were monitored for clinical signs, antibody responses and viral RNA. Nineteen of 20 animals vaccinated with BTV-1 VLPs either alone or in combination with BTV-4 VLPs developed neutralising antibodies to BTV-1, and group-specific antibodies to BTV VP7 (de Diego *et al.*, 2011).

The two viral surface proteins (VP2/VP5) when used together in high doses (100 µg/dose) gave complete protection in sheep against homologous virulent virus challenge. Further vaccination with as little as 10 µg VLPs (consisting of all four major proteins i.e. VP3, VP7. VP2 and VP5) gave long lasting protection (at least for 14 months) against homologous BTV challenge Cross-protection was also achieved depending on the challenge virus and concentration of VLPs used for vaccination (Roy, 2003). Limited vaccination trials with CLPs (containing only two highly conserved internal proteins, i.e. VP3/VP7) gave partial (with slight pyrexia) protection against homologous and heterologous virus challenges (Roy, 2003).

(iii) Cell-mediated immune response

Cell-mediated immunity is evident in BTV infection and there is cross reactivity between viral serotypes (Ghalib *et al.*, 1985). This immunity was described as being a transient heterotypic immunity and is unlikely to provide long term protection against infection (Jeggo *et al.*, 1984; Takamatsu & Jeggo, 1989). Cell-mediated immune response to BTV can probably reduce the spread of virus in the host early after infection, but cannot eliminate the virus completely (Barratt-Boyes *et al.*, 1995).

Passively transferred T-cell enriched thoracic duct lymphocytes from a BTV-infected sheep have been shown to partially protect recipient sheep against infection to either homologous or heterologous serotypes of BTV, confirming that T-cells can mediate cross-protection and that

the effect is not due to B-cells and therefore antibody production (Jeggo *et al.*, 1984). However, cross-reactivity of BTV-infected sheep T-cells (cell line with cytotoxic T-cell activity) against heterologous BTV strains did not correlate with the cross-reactions shown by neutralising antibodies between closely related serotypes using the virus neutralisation assay (Takamatsu & Jeggo, 1989).

Bluetongue virus-specific cytotoxic T cells (CTCs) have been demonstrated in BTV-infected mice (Jeggo & Wardley, 1982a; Jeggo & Wardley, 1982b; Jeggo & Wardley, 1982c) and sheep (Jeggo *et al.*, 1984; Jeggo *et al.*, 1985). By producing a cytotoxic effect in infected cells, CD8+ T-lymphocytes play the most important role in cell-mediated immunity (Barratt-Boyes *et al.*, 1995; MacLachlan, 1994; Schwartz-Cornil *et al.*, 2008). More recently Rojas *et al*., (2011) showed that T-cell responses to BTV are directed against multiple and identical CD4(+) and CD8(+) T-cell epitopes from the BTV-8 VP7 core protein in mice and sheep (Rojas *et al.*, 2011). Infection by the virus leads to perturbations in lymphocyte functions including an increase in the blastogenic response to phytomitogens correlated with viral clearance (Ghalib *et al.*, 1985; Odeon *et al.*, 1997).

Recombinant vaccinia viruses expressing truncated or entire BTV proteins were used to map the location of epitopes recognized by cytotoxic T- lymphocytes (CTL) from Australian merino sheep. The non-structural protein, NS1, was recognised by CTL from all sheep, while VP2, VP3, VP5 and VP7 were recognised by CTLs from only some sheep. The remaining proteins (except for VP1, which was not tested) did not contain CTL epitopes. When truncated genes were used to map the location of CTL epitopes, it was found that sheep often have CTLs that recognise more than one epitope in either the NS1 or VP2 proteins. Overall there was considerable diversity in the CTL recognition patterns in the sheep tested (Janardhana *et al.*, 1999).

Bluetongue virus efficiently induces interferon production *in vitro* and *in vivo* (Foster *et al.*, 1991; MacLachlan & Thompson, 1985). The relative contribution of this response to the clearance of the virus is not well understood, but may play a significant part in non-specific immunity during BTV infection. After BTV infection of bovine foetuses *in utero,* MacLachlan *et al.*, (1984) demonstrated a correlation between interferon titres and the ability to isolate the virus (MacLachlan *et al.*, 1984). A high titre of interferon and the presence of the virus however co-existed in the central nervous system of the foetus. The authors

concluded that interferon did not prevent BTV spread in the foetus. Sheep similarly develop interferon during BTV infection but Jeggo and co-workers (1985) also concluded that the interferon response does not appear to play a major role in disease prevention and recovery (Jeggo *et al.*, 1985).

6. Experimental infections to study reproductive failures associated with BTV infection

Transplacental infection with BTV in sheep and cattle can result in early embryonic losses, abortions, the birth of offspring with severe developmental defects, or the birth of clinically normal appearing offspring that may be viraemic (infectious) and/or test positive for viral RNA in their blood (non-infectious), irrespective of serological status (Osburn, 1994; De Clercq *et al.*, 2008).

The occurrence of transplacental infection and associated teratogenesis in sheep was first reported in California in the early 1950s. Schultz and Delay (1955) reported on the birth of large numbers of "dummy" lambs when pregnant ewes were vaccinated with an egg propagated BTV-10 MLV strain between weeks five and six of gestation. Brains from affected lambs exhibited meningoencephalitis and cavitating lesions in the sub-cortical white matter and cerebellum (Schultz & Delay, 1955). Subsequent experimental infection studies using MLV strains have been instrumental in evaluating the occurrence of transplacental infection in different ruminant species as well as for demonstrating the age dependent severity of foetal malformation. In the case of sheep, experimental infection studies indicated that the most severe lesions (hydranencephaly) occur during the first half of gestation, whereas infection after mid-gestation leads to the development of milder focal lesions (porencephaly). Young and Cordy (1964) reported on the occurrence of a necrotizing meningoencephalitis that progressed to hydranencephaly in 20% of foetuses born to ewes vaccinated at day 40 of gestation (Young & Cordy, 1964). Similarly, sheep foetuses experimentally infected at 50-59 days gestation manifested hydranencephaly and retinal dysplasia at birth. In contrast infection of pregnant ewes at days 70-80 of gestation led to the birth of lambs that demonstrated milder focal lesions (Osburn *et al.*, 1971). Richardson *et al.*, (1985) reported the occurrence of porencephaly, cerebellar dysgenesis and growth retardation in term lambs born to sheep inoculated at 40 and 60 days of gestation (Richardson *et al.*, 1985). In a more recent study Flanagan and Johnson (1995) investigated the effects of infection of pregnant Merino sheep with a BTV-23 MLV strain at five different stages of

pregnancy. In this study, a greater percentage of ewes failed to lamb at birth when infected between 35-43 days gestation (20/36 ewes; 56%), as compared to ewes that were infected at days 109-137 of gestation (0/20; 0%). Three ewes infected at days 35-43 aborted, whereas another two ewes had lambs with hydranencephaly (Flanagan & Johnson, 1995).

Infections in cattle foetuses have been found to cause similar lesions as those observed in sheep, however in earlier studies infection of cattle foetuses could only be initiated following inoculation of foetuses directly through the uterine wall (thereby bypassing the placental barrier) and not following systemic infection of the adults (Parsonson *et al.*, 1987; Roeder *et al.*, 1991). Experimental infection of cattle foetuses demonstrated that the most severe defects (hydranencephaly) occur when foetuses were inoculated between 75-130 days gestation, whereas in contrast foetuses infected after this time demonstrated only cerebral cysts and dilated lateral ventricles (MacLachlan and Osburn, 1983; MacLachlan and Osburn, 1985). Barnard and Pienaar (1976) inoculated two cattle foetuses *in utero* at days 126 and 138 of gestation with a BTV-10 MLV. One foetus aborted at day 262 of gestation while the other one was born alive on day 273. Both foetuses showed marked hydranencephaly (Barnard & Pienaar, 1976). In another study MacLachlan *et al*., (1985) inoculated cattle foetuses at 85-125 days of gestation. At birth infected foetuses demonstrated thin-walled cerebral hemispheres, dilated lateral ventricles, cerebral cysts or the cerebral cortex was replaced by fluid filled sacs (MacLachlan *et al.*, 1985). Similarly, Thomas *et al*., (1986) inoculated three bovine foetuses at days 106, 113 and 122 of gestation with BTV-11. These foetuses spontaneously aborted and demonstrated fluid filled meninges and cerebellums that were reduced in size (Thomas *et al.*, 1986). The age dependent severity of lesions caused by infection of bovine foetuses was demonstrated by Walvogel *et al*., (1992) who inoculated cattle foetuses through the uterine wall with two strains of BTV-11 at both early and late gestation. Both strains were able to cause severe neurological lesions (hydranencephaly) when foetuses were inoculated at days 120 of gestation. Foetuses inoculated later during gestation (243 days) in contrast were either born clinically normal and/or demonstrated mild encephalitis (Waldvogel *et al.*, 1992a; Waldvogel *et al.*, 1992b).

The age dependency of the severity of cerebral malformation occurs as a result of differences in cell susceptibility/tropism of BTV in the developing brain as well as the immune status of the foetus at the time of infection (MacLachlan *et al.*, 2000; Osburn, 1994). Bluetongue virus shows a tropism for neuronal and glial pre-cursor cells that populate the sub-ependymal

region of the brain prior to their migration to the cortical white matter and cerebral cortex. Virus-mediated destruction of these cells at early gestation prevents the formation of the cerebral hemispheres (hydranencephaly). In contrast these cells are less susceptible to BTV infection following their migration in the cerebral cortex, resulting in milder focal cavitating lesions (porencephaly) (MacLachlan *et al.*, 2000; Osburn, 1994). The immune status of the foetus may also influence the ability of the virus to spread and cause lesions in the brain. Passive transfer of antibodies does not occur in ruminants across the placental barrier. The foetal immune system further only becomes competent for the production of neutralising antibodies around mid-gestation (95 days in foetal lambs; 175 days in foetal calves). Infection of the foetus during early gestation therefore essentially allows the virus to replicate and spread unhindered, whereas virus spread during the second half of gestation is curtailed by the developing immune system (Osburn, 1994). The precise mechanism by which foetal infection occurs with BTV is not known. It has been speculated that the virus may be able to cross the placenta either through transfer of infected monocytes across the placental barrier or direct viral transfer across the trophoblast (Osburn, 1994). A recent study has demonstrated that zonae pellucidae-free bovine blastocysts are susceptible to BTV-8 infection *in vitro*, and that infection induces apoptotic cell death of blastomeres. The study concluded that infection of early embryos/blastocysts *in utero* may contribute to early embryonic losses associated with BTV-8 infection in cattle (Vandaele *et al.*, 2011).

Earlier observations that certain strains of BTV are able to cross the placenta raised the question as to whether BTV is able to establish an inapparent, persistent carrier state following transplacental infection. This is of particular concern due to the role that a carrier state could play in the trans-seasonal persistence of the virus in temperate regions as well as the possibility that the inadvertent transport of "healthy appearing" persistently infected offspring can result in the introduction of the virus into unaffected regions (MacLachlan & Osburn, 2006). Leudke *et al.*, (1977) reported on the apparent development of immune tolerance and persistent infection of calves whose dams were infected with BTV-11 by *Culicoides* bites at day 60-120 of gestation. It was reported that it was possible to isolate virus via blood auto grafting in sheep from several "healthy appearing" calves for months to years after birth (Luedke *et al.*, 1977). Several follow-up studies designed to confirm these findings however failed to yield similar results (Parsonson *et al.*, 1987;Roeder *et al.*, 1991;Thomas *et al.*, 1986), which has raised questions as to the correctness of the findings reported by Luedke *et al.*, (1977). Currently it is accepted in OIE member countries that

immuno-tolerance and/or persistent BTV infections does not occur (Darpel *et al*., 2009; De Clercq *et al*., 2008; Walton, 2004).

Historically transplacental infection with BTV has been associated mainly with infections caused by MLV strains, and rarely with infection caused by wild-type strains of the virus (Kirkland & Hawkes, 2004). However the wild-type BTV-8 strain that caused the 2006-2008 outbreak of BT in northern Europe was particularly noteworthy for its ability to cross the placenta of sheep and cattle at a high frequency. Field evidence for transplacental transmission of BTV-8 among sheep and cattle in Europe in the field has been confirmed with the aid of PCR positive test results that were obtained from tissues of aborted foetuses, the detection of BTV-8 specific neutralizing antibodies in the serum of pre-colostral calves and lambs, the occurrence of congenital deformities that suggest that transplacental infection had occurred, and q/RT-PCR positive test results from blood samples that were taken from animals that were born during the vector-free period of the year (Saegerman *et al.*, 2010a). A study in Belgium reported that BTV RNA was present in 41% and 18.5% of aborted foetuses taken from dams with or without a suspicion of BT infection respectively, while 11% of calves born with clinical signs suspicious of BT tested positive for the virus (De Clercq *et al.*, 2008). Transplacental transmission rates in cattle in the Netherlands and United Kingdom have also been reported to be high. Incidence rates of 16.2% was recorded in 10-day-old new-born calves sampled from dairy herds in the Netherlands in the first quarter of 2008, whereas transplacental transmission rates of 33% were recorded in calves in the United Kingdom amongst dams infected during 2007-2008 (Darpel *et al.*, 2009).

The ability of BTV-8 to be transmitted vertically in Europe has been investigated in experimental transmission studies in cattle, sheep and goats. Backx *et al*., (2009) demonstrated that a real-time RT-PCR positive but seronegative calf can be born to a dam that was infected with BTV-8 at 8 months of gestation. This study also demonstrated that calves could be infected with BTV-8 through the oral ingestion of virus contaminated colostrum (Dal Pozzo *et al*., 2009) The first experimental evidence that BTV-8 is able to cross the placenta in sheep was demonstrated by Worwa *et al*., (2009) (Worwa *et al.*, 2009). In this study it was found that a foetus that was harvested from a ewe infected at 11 weeks of gestation tested positive for BTV RNA when sacrificed at 14 dpi. Another ewe that was infected at 11 weeks of gestation and which carried the foetus full term also gave birth to a lamb that displayed branchygnathia inferior. A subsequent study confirmed that sheep are

highly susceptible to transplacental infection with BTV-8, with reported infection rates varying between 30% and 69% in ewes infected at early (day 40-45) and mid-gestation (70-75 days), respectively (van der Sluijs *et al.*, 2011).

The first evidence of transplacental infection in four goats infected with BTV-8 at 62 days of gestation has recently been reported in a study conducted in the authors' laboratory. In this study, viral RNA (segment 5) could be detected by real-time RT-PCR in blood and tissue samples from three foetuses harvested from two goats at 43 dpi. Viral RNA was also detected in placental tissue from two additional goats at 13-25 dpi, although infection of two foetuses carried by these animals could not be established. The majority of foetuses (5/6) demonstrated lesions that may have been associated with transplacental infection with BTV, which included haemorrhaging in the pulmonary artery of one foetus (Coetzee *et al.*, 2013). In another study in nine goats that were experimentally infected with BTV-8 at 61 days of gestation, BTV-8 was detected by real-time RT-PCR and virus isolation in blood and spleen samples of 3/13 foetuses collected from adults at 21 dpi (day 82 of gestation). In a second experiment, 10 goats that were infected with BTV-8 at 135 of gestation gave birth to 11 kids. Bluetongue virus serotype-8 could not be detected by real-time RT-PCR in blood nor could gross lesions be demonstrated at birth (Belbis *et al.*, 2013). Together these two studies suggest that goats are more susceptible to BTV-8 transplacental infection at early gestation. Interestingly in neither of these two studies could cerebral malformations of infected foetuses be demonstrated.

It remains uncertain as to why the European BTV-8 field strain was able to cross the placenta in ruminants. The mutation/s that are incorporated into the viral genome during its adaptation to embryonated chicken eggs/cell culture and that facilitate transplacental infection have not been identified (Kirkland & Hawkes, 2004). These mutations either arose spontaneously in the European BTV-8 field strain by genetic drift and/or the BTV-8 strain may have acquired the ability to cross the placenta by reassortment with a circulating vaccine and/or unknown wild-type strain (Maan *et al.*, 2008; Anon, 2011). In the absence of comprehensive whole genome sequence data for MLV strains it has however, not been possible to conclusively demonstrate that the European BTV-8 strain is a reassortant with an MLV strain.

Transplacental infection in the European context with BTV-8 has had severe economic consequences, particularly due to movement restrictions that were placed on pregnant

heifers/ewes. In 2008, BTV-8 seropositive but PCR negative heifers imported into Northern Ireland from the Netherlands gave birth to three healthy looking calves of which two were BTV RT-PCR positive and one was viraemic as demonstrated by virus isolation during the vector-free period of the year. These observations highlighted the possibility of introducing BTV-8 into new regions through the importation of seropositive pregnant animals (Menzies *et al.*, 2008). This initially led to a blanket export ban of pregnant heifers and ewes and later a requirement for pre-testing under quarantine conditions and/or vaccination before animal export.

Experimental infection studies have been conducted in order to investigate seminal shedding and the possibility of transmitting BTV through infected germ plasm. Earlier experimental infection studies in bulls suggested that BTV is not persistently secreted in semen. Instead BTV was found intermittently in semen, particularly in older bulls, but only in association with contaminating blood cells during infectious viraemia (Kirkland *et al.*, 2004). Bluetongue virus contaminated bull semen was further demonstrated to be able to transmit BT to receptive heifers; however, infection of these animals failed to transmit the virus to their offspring (Bowen *et al.*, 1985b). In more recent studies it has been demonstrated that RNA of the European strain of BTV-8 is detectable in the semen of a high proportion of BTV infected rams (Leemans *et al.*, 2012) and bulls (Vanbinst *et al.*, 2010) by real-time PCR. In rams, viral RNA could be detected for up to 116 days post infection, whereas 48 of 89 semen samples taken from 19 BTV-8 infected bulls between August 2007 and February 2008 tested positive. Virus isolation was also successful from 4 samples from bulls, indicating the presence of infectious virus. It still remains to be clarified whether BTV-8 contaminated semen is infectious to recipient heifers/ewes.

The possibility of transmitting BTV through embryo transfer from infected donors to non-infected recipient animals has also been the subject of intensive study. Experimental studies designed to investigate whether pre-implantation embryos recovered from viraemic cows could transmit BTV to seronegative recipients have failed to demonstrate transmission of the virus (Acree *et al.*, 1991;Bowen *et al.*, 1985a), whereas similar results have been obtained in experimental studies in sheep (Hare *et al.*, 1988;Singh *et al.*, 1997). It is currently accepted that the transmission of BTV via embryo transfer or the use of BTV infected semen represents a negligible risk for the transmission of the virus, as long as validated procedures for embryo washing are followed and semen is tested for the virus prior to export (Al Ahmad

et al., 2011; Al Ahmad *et al*., 2012;Venter *et al*., 2011;Wrathall *et al.*, 2006).

7. Experimental infections to study the pathogenesis of bluetongue

Experimental infection studies in ruminants have been instrumental in delineating the cellular and organ tropism of BTV as well as for determining the sequential dissemination of the virus throughout the ruminant host following infection. These studies initially focused on investigating the spread of virus following experimental infection (via the skin), by evaluating the appearance of viral antigen in different tissues of sequentially euthanased animals using immunological staining methods, as well as the appearance of virus in blood and tissue using virus isolation. Experimental infection studies in sheep (Pini, 1976) and cattle (Barratt-Boyes & MacLachlan, 1994), led to the development of the current BT pathogenesis model. These studies indicated that following cutaneous instillation through the skin the virus travels to local draining lymph nodes, via efferent lymph, where low level virus replication occurs. The importance of the lymphatic system in the early replication of the virus was highlighted in particular in calves in which the flow of efferent lymph was interrupted by indwelling cannulas. In these animals the interruption of efferent lymph flow from prescapular lymph nodes was able to delay the onset of viraemia from 3 to 7 days. Following primary replication in local draining lymph nodes, the virus is disseminated at a low level via blood in association with infected leukocytes to the spleen, thymus, tonsils and other lymphatic tissues where secondary replication takes place. This is followed by a high level, cell associated viraemia, during which the virus spreads to tissues throughout the body.

Ultra-structural studies have indicated that BTV becomes associated with cell membrane invaginations of erythrocytes during viraemia (Brewer & MacLachlan, 1992). Infectious virus is therefore able to circulate in the presence of neutralising antibodies for several weeks (Barratt-Boyes & MacLachlan, 1994; Brewer & MacLachlan, 1994). Experimental infection studies have further indicated that BTV RNA may be detected in blood from sheep and cattle, even when virus can no longer be isolated and blood is no longer infectious to the vector. The duration of PCR positivity for BTV has been documented to be prolonged in cattle and sheep (111-222 days post infection), appears to be related to the half-life of the ruminant erythrocyte, and is longer in cattle than in sheep (van Rijn *et al*., 2012;Bonneau *et al*., 2002; Barratt-Boyes and MacLachlan, 1995). The length of infectious viraemia has been investigated extensively in experimentally infected sheep and cattle (Bonneau *et al.*, 2002;

Singer *et al.*, 2001). Based on these findings, member countries of the World Organisation for Animal Health have adopted a resolution that sets the maximum infective period of BTV infection in ruminants as 60 days (OIE, 2004).

Bluetongue virus demonstrates a tropism for a variety of cell types, including dendritic cells, mononuclear phagocytic cells, activated lymphocytes and endothelial cells (Drew *et al.*, 2010b; Hemati *et al.*, 2009; Mahrt & Osburn, 1986;Stott *et al.*, 1990). A recent study utilised con-focal microscopy, together with immuno-labelling of non-structural protein 2 (NS2) and viral protein 7 (VP7), in order to distinguish between cells in which the virus was merely present and cells in which the virus was actively replicating. Five Dorset Poll sheep were infected with BTV-2, using reconstituted freeze-dried sheep blood that contained a virus that had not previously been passaged in tissue culture. Each animal received 1.5 mL of the inoculum subcutaneously into the left side of the neck and 0.5 mL intradermally into the right inner thigh. In this study, virus replication could be demonstrated in endothelial cells and agranular leukocytes (lymphocytes, monocytes/macrophages and/or dendritic cells) in several tissues from days 3-9 post infection. The study also demonstrated the presence of virus replication in association with the microvascular endothelium of the tonsils and skin. Prior to this study, these organs had not been implicated as key sites of infection and replication of the virus (Darpel *et al.*, 2012). Another study indicated that conventional dendritic cells appear to be initial targets for BTV replication in the skin of experimentally infected sheep, and that these cells represent the primary cell type responsible for the initial dissemination of the virus from the skin to local draining lymph nodes. This study also indicated that BTV infection of dendritic cells had no adverse impact on their physiology *in vitro*, but appeared to enhance their viability (Hemati *et al.*, 2009).

Bluetongue associated lesions occur mainly as a result of virus-mediated damage to endothelial cells lining the vasculature. Damage to these cells (characterized by nuclear changes, cytoplasmic vacuolation/granulation, cell rounding, hypertrophy and lysis) manifest as the main pathological changes of BT that include vascular thrombosis, tissue infarction (necrosis), haemorrhage and vascular leakage (MacLachlan *et al.*, 2009). Immuno-staining has indicated that endothelial cell infection of tissues in experimentally infected sheep appears to be relatively sparse and transient (Darpel *et al.*, 2012; Mahrt & Osburn, 1986). Virus-mediated damage to endothelial cells is therefore not thought to be the sole mechanism involved in the pathogenesis of the disease. Recent studies suggest that inflammatory and

vasoactive mediators secreted by BTV infected cells in response to infection play an additional role. Specifically it has been suggested that direct virus-mediated damage of endothelial cells are responsible for the vascular thrombosis, tissue infarction and necrosis that is seen during the acute phase of the disease, whereas the widespread oedema/haemorrhaging that is seen during the terminal phase of the disease is related to endothelial cell contraction and vascular leakage caused by the paracrine activity of pro-inflammatory and vasoactive mediators (Drew *et al.*, 2010a). Treatment of bovine endothelial cells with either partially purified virus or the pro-inflammatory cytokine TNF-α (Tumour Necrosis Factor) *in vitro* has demonstrated that BTV infection results in a delayed reduction of cell culture monolayer integrity as a result of BTV induced cell death, whereas the treatment of cells with TNF-α results in a rapid loss of cell monolayer integrity due to the redistribution of VE-cadherin (a cellular protein involved in cell to cell adhesion), but without associated cell death (Drew *et al.*, 2010a). *In vivo* studies in experimentally infected goats combined with immuno-histochemical staining of lesions for the presence of virus and certain cytokines (TNF-α and IL-1α), have further demonstrated a potential association between the development of lesions, virus infection and the secretion of pro-inflammatory cytokines (Sanchez-Cordon *et al.*, 2012).

Cattle and sheep demonstrate differences in the clinical presentation of BT. Clinical signs in sheep can vary from subclinical to overt, whereas infections in cattle are usually subclinical (infections caused by the European BTV-8 strain being an exception) (Dal Pozzo *et al.*, 2009; Darpel *et al.*, 2007). Several studies have contributed to attempts to elucidate the underlying basis for the disparate clinical presentation of BT between sheep and cattle. With regards to the clinical presentation of BT in the two host species, it has been shown that (a) the susceptibility of endothelial cells to BTV infection differs between sheep and cattle and that (b) the level of expression of inflammatory and vasoactive mediators as well as cell surface adhesion molecules differs between ovine and bovine endothelial cells (DeMaula *et al.*, 2002a; DeMaula *et al.*, 2002b). Upon BTV infection of bovine endothelial cells, a marked increase in the expression of pro-inflammatory and vasoactive mediators (IL-1, IL-6, IL-8, cyclooxygenase-2 and inducible nitric oxide synthase) and cell adhesion molecules (E-selectin, GR antigen and MHC-2) occurs as compared to ovine cells (DeMaula *et al.*, 2002a; DeMaula *et al.*, 2002b). Bovine endothelial cells further secrete a higher level of anti-thrombotic prostacyclin following infection than ovine cells (DeMaula *et al.*, 2001). Experimental studies in sheep and cattle have confirmed that the ratio of thromboxane to

prostacyclin that is secreted by the two host species is higher in BTV infected sheep than cattle (DeMaula *et al.*, 2002a). These observations suggest that cattle are better able to regulate platelet aggregation/thrombosis following BTV infection than sheep.

8. Experimental infections to study the potential risks associated with the genetic drift, reassortment and recombination of BTV

Bluetongue virus evolves though a complex process of antigenic drift (point mutation and deletions) and shift (reassortment and intragenic recombination) coupled with founder effect and positive/negative selection (Balasuriya *et al.*, 2008; Bonneau *et al.*, 2001; He *et al.*, 2010; Samal *et al.*, 1987b). Bluetongue virus evolutionary processes have over time led to evolution of distinct serotypes/strains of the virus in different epidemiological systems (episystems). Viruses that occur in different episystems have undergone unique adaptations for spread/persistence in their ecological niches and may therefore differ in regards to their phenotypic characteristics. As an example it has been reported that virulence differs markedly between viral strains belonging to different serotypes in South Africa, Australia, Europe and the United States (Dal Pozzo *et al.*, 2009; Gibbs & Greiner, 1994; Hooper *et al.*, 1996). Anecdotal reports further suggest that BTV strains in South Africa may differ in their pathogenic and epidemic potential (Verwoerd & Erasmus, 2004). The adaptation of the virus to embryonated chicken eggs/cell culture may further lead to the introduction of unwanted phenotypic characteristics by genetic drift, such as the ability of the virus to cross the ruminant placenta, and/or perhaps an increased tendency to be secreted in the semen of infected males (Kirkland & Hawkes, 2004; Leemans *et al*., 2012; Vanbinst *et al*., 2010).

A major concern that has been highlighted when BTV spreads into new regions or when an attenuated vaccine strains persist in the field following vaccination campaigns is that the virus may reassort/recombine with local field strains, resulting in dramatic changes in the phenotype of the virus over relatively short evolutionary time periods (i.e. genetic shift). In particular there is a concern that reassortment and/or recombination may lead to rapid changes in the virulence, pathogenicity and transmissibility characteristics of the virus (Coetzee *et al.*, 2012). Reassortment/recombination between virulent wild-type and vaccine strains of the virus may for example hypothetically lead to the emergence of a virus that could incorporate the high virulence of a field strain with the ability of a vaccine strain to cross the placenta. Further reassortment between exotic and indigenous strains could

potentially facilitate the maintenance of genome segments from exotic strains by affecting the transmissibility of the virus by indigenous vector species (Shaw *et al*., 2013). Prior studies have indicated that genome segment reassortment appears to be highly flexible and may potentially involve any of the genome segments (Shaw *et al.*, 2013). As previously mentioned, information of the genome segments/gene products and/or specific genetic markers that are involved in influencing the phenotype of BTV is lacking (Caporale *et al.*, 2011; Darpel *et al.*, 2011; Huismans & Howell, 1973; Riegler, 2002). It is therefore currently extremely difficult to predict what effect reassortment and/or recombination will have on the phenotypic properties of parental strains.

The risk of reassortment/recombination has been highlighted during recent outbreaks of BTV in Europe, where several reassortant/recombinant strains of both vaccine and wild-type origin have been isolated from the field. The isolation of a double reassortant field strain of BTV-16 that contained a genome segment 2 (VP2) derived from a live-attenuated BTV-16 vaccine strain and a segment 5 (NS1) derived from a live-attenuated BTV-2 vaccine strain was reported in Italy in 2002 (Batten *et al.*, 2008). In late 2008, a BTV-6 strain was detected for the first time in Europe in the eastern Netherlands (Overjissel and Gelderland Provinces) and later in adjacent parts of Germany (Lower Saxony) in cattle that displayed mild non-specific clinical signs of BT. Sequencing of the virus indicated that segment 1 to 9 showed high nucleotide sequence identity to a live-attenuated BTV-6 vaccine strain, however this virus also contains a segment 10 (NS3/A) that was highly similar to a live-attenuated BTV-2 vaccine strain (Maan *et al.*, 2010). More recently a multi-reassortant composed of genome segments of field strains of BTV-1 and BTV-8 has been isolated in France in 2008 (Shaw *et al.*, 2013). With regards to recombination, the isolation of viral strains containing mosaic sequences [segments 1 (VP1), 4 (VP4), -5 (NS1),7 (VP7) and 10 (NS3/A)] of both wildtype and vaccine origin and derived from viral lineages isolated from Italy, Greece, the Netherlands, France and Turkey have been reported (He *et al.*, 2010).

Studies to investigate BTV reassortment have mostly focused on investigating the kinetics of the reassortant process using different *in vitro* and *in vivo* systems (Ramig *et al.*, 1989; Samal *et al.*, 1987a; Samal *et al.*, 1987b). Only a few studies have attempted to predict what effects reassortment could have on the manifestation of clinical signs. These studies mainly focused on evaluating the effects of reassortment by using cell culture based methods (i.e. replication kinetics and/or plaque morphology) with only a few experimental infection studies having

been conducted in animals (i.e. mice and/or ruminants) Carr *et al*., 1994; Shaw *et al*., 2012; van Rijn *et al*., 2012; Waldvogel *et al*., 1986; Waldvogel *et al*., 1987; Waldvogel *et al*., 1992b; Waldvogel *et al*., 1992a). No study reports are currently available that have attempted to address the potential effects of recombination on the phenotype of the virus.

In vivo examples of genetic reassortment resulting in the alteration of either virulence and/or pathogenicity have largely been limited to the study of naturally occurring VP5 reassortants of BTV-11 named UC-2 and UC-8 that were isolated from the field in the USA. The UC-2 and UC-8 strains shared the same VP2 segments, but differed in regards to their VP5 segments. The UC2 strain derived its genome segment 6 (VP5) from a live attenuated BTV-11 vaccine strain, while UC-8 derived its segment 6 from a live attenuated BTV-10 vaccine strain (Osburn, 1994). These strains demonstrated differences in their pathogenicity characteristics in experimentally infected mice and bovine foetuses (Carr *et al.*, 1994; Waldvogel *et al.*, 1986; Waldvogel *et al.*, 1987; Waldvogel *et al.*, 1992a; Waldvogel *et al.*, 1992b). Recently Shaw *et al*., (2013) evaluated the virulence properties of selected BTV-1/8 mono-reassortant strains in experimentally infected $IFNAR^{(-/-)}$ mice. In this study the exchange of genome segments between the two serotypes did not appear to result in significant differences in *in vivo* virulence (Shaw *et al.*, 2013). Experimental infection studies in cattle and sheep with the BTV-6 vaccine reassortant isolated in the Netherlands in 2008 has further indicated that the virus had an avirulent phenotype (van Rijn *et al.*, 2012).

It should be noted that a reverse genetics system for BTV has recently been developed that allows for the *de novo* generation of BTV strains (Boyce *et al.*, 2008). The technology, together with experimental *in vitro* and *in vivo* studies (including experimental infection studies in mice, ruminants and insects) will undoubtedly prove to be an invaluable tool for delineating the virulence/pathogenicity/transmissibility markers of BTV in future. The generation of novel reassortant strains between parental strains with different phenotypic characteristics (for example between virulent and avirulent strains, or strains that are able to cross the placenta and those that are unable to do so) may for example assist in the identification of the genome segments/gene products and mutation/s responsible for virulence and/or transplacental infection. Such studies may in the long run lead to a better understanding of the effects and risks of BTV reassortment and/or recombination, and possibly to the development of molecular tests to detect particular phenotypic traits. Interestingly reverse genetics technology for BTV combined with reassortment forms the

basis for the development serotype-specific disabled single cycle infectious (DISC) vaccines that can easily be tailored to outbreaks caused by different serotypes/strains. This can be done by exchanging the outer capsid genes that encode VP2 and VP5 on an attenuated genetic backbone (van Gennip *et al.*, 2012; Celma *et al.*, 2013).

9. Animal welfare considerations with experimental BTV infections

Animal experiments always have serious ethical and welfare implications. The issue of pain and distress in animals subjected to experimental infection concerns both the general public and researchers. The outcome of the experiment has to justify the adverse effects on the animal. A researcher performing such experiments must be prepared to explain and justify why a particular study was conducted. The arguments for permitting animal experiments in BT research will differ since the aims of the studies vary from basic research on pathogenesis to testing potential vaccines.

Ruminants are the natural host species in which BT disease occurs and are therefore the preferred model in which to conduct experimental infection studies. Infection studies using ruminants are however expensive and time consuming and limited numbers of animals are therefore generally included. In addition, the complexity of the biological system of the whole animal gives unpredictable results and high variability, and often low repeatability. Inbred mice are far easier and cheaper to work with. Extrapolation of findings in mice to ruminants must however be done with care due, to differences in the biology between mice and ruminant species (Calvo-Pinilla *et al.*, 2009; Franchi *et al.*, 2008; Narayan & Johnson, 1972).

Animal experiments are regulated by national and international laws and regulations. Most agencies responsible for setting standards for the care and use of experimental animals require investigators to consider the justification of the experiment and to implement the concept of the 3Rs (Reduction, Replacement and Refinement). The general principle of the 3Rs namely “Reduction, Replacement and Refinement” was developed many years ago and has become widely accepted as ethical principles (Balls *et al.*, 1995). The 3Rs have been defined as "all procedures which can completely replace the need for animal experiments, reduce the numbers of animals required, or diminish the amount of pain or distress suffered by animals in meeting the essential needs of man and other animals” (Smythe, 1978). In

general, the 3R measures can be implemented to improve the welfare of animals. The 3Rs also contribute to the quality of research findings through improved study design, reduced variability and increased statistical power. The reader may consult the NORECOPA (Norwegian Consensus Platform for Replacement, Reduction and Refinement of animal experiments) website (http://www.norecopa.no/sider/tekst.asp?side=19) for detailed information on the rationale behind and implementation of the 3Rs. Special guidelines have further been developed to improve the study design, analysis and reporting of research using animals, the so-called ARRIVE guidelines (Killkenny *et al*., 2010). These guidelines are endorsed by an increasing number of scientific journals and may be consulted at the following web URL: (http://www.nc3rs.org.uk/downloaddoc.asp?id=1206&page=1357&skin=0). A summary of the principles behind the 3Rs are provided below:

(i) Replacement

Replacement refers to the replacement of animal experiments with non-animal alternatives, which can vary from computer models to less sentient animals or cell cultures. Ideally, all possible laboratory investigations should be performed before animal experiments are set up. Laboratory conditions are more controlled and the variability factors that complicate live animal research are reduced. However, *in vitro* testing can only partly provide a surrogate for *in vivo* infection. For BT, it seems to be difficult to find *in vitro* parameters that predict *in vivo* properties adequately. Extrapolation from *in vitro* parameters to *in vivo* characteristics are therefore challenging for BTV (Caporale *et al.*, 2011; Franchi *et al.*, 2008; Shaw *et al.*, 2013).

(ii) Reduction

Reduction implies a decrease in the number of experimental animals without loss of information. This may be achieved through good experimental design and/or by controlling variation. The variation can be decreased by the use of genetically homogenous animals, by using controlled environmental conditions and/or by adhering to strict management procedures.

The natural hosts for BTV are resource-demanding to buy and accommodate. Therefore the sample size is usually small and the exact number is chosen for practical/economic reasons. Particularly when pregnant ruminants are used, the number is limited to a minimum. The use of low numbers of animals may be unsuitable for statistical analysis, and the result are therefore often only useful to provide proof of principle. When mice models are chosen, the result will be less valid than for the natural host, but it does provide an opportunity to increase the sample size. Traditionally, a standard parallel design has been used in mice experiments (Calvo-Pinilla *et al.*, 2009; Franchi *et al.*, 2008). The main advantage is that it is well established, and is easy to plan and manage. More sophisticated statistical designs are however available where the number of mice can be reduced without loss of statistical power (Festing *et al.*, 2002; Festing & Altman, 2002).

(iii) Refinement

Refinement refers to a change in scientific procedures and animal husbandry to minimize suffering, pain, stress and distress that the animals may experience. Refinement enables healthy animals with normal social behavior that also results in less variability with improved results. Relevant factors to consider will vary with the animal species and type of study. This could include appropriate use of anaesthetics, analgesics and other therapeutic measures, and the refinement of husbandry to improve the well-being of the animals (such as the use of environmental enrichment including litter and toys). Humane endpoints should further always be established to avoid unnecessary suffering for the animals.

(iv) Implementation of the 3Rs in a murine model

In a study investigating viral virulence properties, suckling mice were inoculated via the intracranial route with different reassortant strains of BTV (Coetzee *et al*, manuscript in preparation) and the 3R´s applied at various stages.

1. In the first instance *in vitro* studies were conducted. These studies were aimed at measuring differences in the replication kinetics, virus-induced cytopathogenicity and the degree and mechanism of cell death. The possibility of evaluating *in vivo* properties in ruminants was considered, but would have led to a lack of statistical power due to low animal numbers. Another animal model was therefore required and intracranial

inoculation of suckling mice therefore conducted (Caporale *et al.*, 2011; Franchi *et al.*, 2008).

2. In an effort to find a better statistical method than a standard parallel design, a "Response Surface Pathway Design" was chosen. The model was developed to reduce the number of animals included in LD_{50} studies, without loss of information. The model has been used in toxicological and pharmacological studies, and is suitable for LD_{50} studies in virology (Dew *et al*., manuscript in preparation). In total, the number of mice used in the design is approximately three times less than standard models that are traditionally used in LD_{50} studies

3. Anaesthesia and analgesia was used when the mice were inoculated. It is not clear how commonly this is used in BT research due to a lack of documented information.

4. Randomisation was applied to avoid cage and litter variables.

5. The randomisation required that newborn mice be labeled. Different methods were tested, but no single method was found to be more reliable than toe clipping. Toe clipping is considered to be a painful procedure. Therefore both systemic and topical analgesics were applied to reduce the pain and discomfort.

6. Refinement of husbandry was highlighted. Mothers housed in cages with newborn mice are sensitive to stress, which commonly leads to cannibalism. The use of litter, material for nesting, chewing sticks and the control of environment conditions (light, sound, temperature etc.) were emphasized.

7. Close monitoring was strictly followed to be able to identify mice with signs of disease as soon as possible (mice were observed twice daily in the mornings and afternoons). Conversely, the regular opening of the cages and disturbing of the mice were regarded as a stress factor. The solution was to check all animals twice a day, but to avoid disturbances by not taking the animals out of the cage.

8. Clearly defined humane endpoints were included in the study protocol. This included euthanisation when mice ceased to nurse, the manifestation of neurological signs or any signs of poor health and/or abnormal behavior.

9. The inclusion of information on the time of death/euthanasia was included in the statistical model.

10. All mice that were withdrawn from the study or completed the study were euthanased by means of intra-abdominal injection of sodium pentobarbitone.

11. The ARRIVE guidelines were followed for presentation of the study results.

(v) Implementation of 3Rs in a caprine model

Since transplacental infection is a crucial factor in BT pathogenesis and epidemiology, experiments on pregnant animals of different species are necessary. Experimental infection with BTV in pregnant animals can be regarded as ethically very challenging, but no other models are available that can mimic the natural situation. In a study by Coetzee *et al* (2013) on transplacental infection of BTV-8 in goats (Coetzee *et al.*, 2013), the 3Rs were implemented by, among others, the following considerations:

1. The choice of model and animal species; no *in vitro* models or other mammalian species that could mimic the situation in pregnant goats were available. The number of goats was chosen for practical and economic reasons, together with knowledge of fertility and abortion rates. Saanen goats were chosen, since it represented a European breed and BTV-8 exerted its effects on ruminants in European countries.

2. The goats were housed in groups to avoid isolation (goats are highly social).

3. Close monitoring by experienced laboratory animal technologists was done twice daily during the acute phase of the disease, and more often when deemed necessary.
4. Animals with fever and/or signs of pain or discomfort (such as frequent displacement of weight on the limbs, grunting or grinding of teeth) were given anti-inflammatory drugs. Animals with mild lesions and prolonged pyrexia were treated with antimicrobial drugs for secondary bacterial infections.

5. A clear definition of humane endpoints was used. Clear defined endpoints included animals which were unable to rise or take sustenance for one day, animals with severe oedema of the head and neck, or that displayed severe necrosis of the oral mucosa and/or dyspnoea.

6. The method for euthanasia and the experimental endpoints were clearly described.

10. Competing interests

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

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