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Recent Developments in Immunoprophylaxis, Diagnosis and Epizootiology of Bluetongue Virus in Germany

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“I begin to be almost sorry I was born so soon, since I cannot have the happiness of knowing what will be known 100 years hence.” (Benjamin Franklin)

1 Introduction

Bluetongue virus (BTV) is the type species of the genus *Orbivirus* in the family *Reoviridae* (Van Regenmortel, 2003; Mertens *et al.*, 2004b). Currently, there are 24 recognized serotypes (BTV-1 to -24) and an unconfirmed 25th, "Toggenburg orbivirus" (TOV) (Hofmann *et al.*, 2008). BTV is almost exclusively spread by *Culicoides* spp. biting midges (*Diptera*) and occurs worldwide. All 24 serotypes can cause bluetongue disease (BT), a non-contagious hemorrhagic disease of domestic and wild ruminants and camelids with no known zoonotic potential (Verwoerd and Erasmus, 2004).

BT primarily affects sheep, but clinical disease in cattle and deer does occur. It can have considerable economic impact, both directly by deaths and decreased productivity and indirectly by trade losses through animal movement restrictions (Verwoerd and Erasmus, 2004). BT is notifiable to the World Organisation for Animal Health (OIE, 2006) and to veterinary authorities in many countries, including Germany (TierSeuchAnzV, 2009; BlauzungenV, 2009).

While bluetongue had long been considered exotic to Europe, repeated incursions and extensive circulation of several serotypes in the Mediterranean and, recently, in Central Europe have arguably made it an European disease (Wilson and Mellor, 2008). The introduction of BTV-8 to Central Europe in 2006 marked the begin of an unprecedented epizootic with severe disease in livestock and financial losses in the hundreds of millions. The EU and Switzerland launched a mass vaccination campaign as soon as inactivated vaccines became available in 2008, the largest such campaign since the end of foot-and-mouth vaccination in the 1990s. These vaccines, however, had not yet obtained EU marketing authorization. German authorities considered the data provided by the manufacturers insufficient for a compulsory vaccination campaign and commissioned independent studies to evaluate the safety and efficacy of the vaccines.

This work reproduces the results of the efficacy study and a follow-up investigation of protection one year after vaccination. The possible implications of inactivated vaccines for BTV diagnosis by real-time RT-PCR were examined in an animal experiment, whose results are included here as well.

While the BTV-8 epizootic was still ongoing, BTV-6 appeared in northwestern Europe in late 2008. The results of a field survey to investigate its prevalence in Germany and related animal experiments are presented, together with serotype-specific real-time RT-PCR assays that were used in the field survey and are now deployed in BTV routine diagnostics.

2 Literature review

2.1 Taxonomy, virion properties and structure

Bluetongue virus, together with the closely related species *African Horse Sickness virus* (AHSV) and *Epizootic Hemorrhagic Disease virus* (EHDV) belongs to the genus *Orbivirus* (comprising at least 20 species overall) in the family *Reoviridae*. The reovirus family includes vertebrate, arthropod and plant pathogens (Mertens *et al.*, 2004b). Unique among reoviruses, all orbiviruses are arthropod-borne viruses (arboviruses), maintained in nature through transmission between susceptible vertebrate hosts by blood-feeding arthropods. The term "arbovirus" itself has no taxonomic significance, merely describing the mode of transmission of viruses of many families and genera. With few exceptions (such as *African Swine Fever virus*), most arboviruses are RNA viruses (Hart, 2001).

The non-enveloped, three-layered icosahedral BTV capsids have a diameter of 86 nm and a relative molar mass of about 10.8×10^7, 12% of which is genomic RNA (cores: 6.7×10^7 and 19.5%, respectively). In the presence of protein, the virus is stable at a temperature of 4 °C, or even 20 °C. Generally, infectivity remains constant between pH 6.5 and 8, but decreases markedly outside of that range (Erasmus, 1990). For low pH, this is probably related to the loss of outer capsid proteins, a phenomenon that is also relevant in the replication cycle. Due to the lack of a lipid envelope, orbiviruses are relatively resistant to solvents and detergents, but readily inactivated by acidic disinfectants, sodium hypochlorite (bleach) and iodophors (Howell and Verwoerd, 1971). Infectivity is abolished at a pH of less than 3 and temperatures exceeding 60 °C. While freezing reduces infectivity by about 90%, it then remains stable at -70 °C, but not at -20 °C (Mertens *et al.*, 2004b).

Like all reoviruses, BTV has a segmented, linear double-stranded RNA genome. Orbivirus genomes have ten segments, with exactly one copy of each segment per particle (Mertens *et al.*, 2004b). Owing to the segmented genome, simultaneous infection of cultured cells, vertebrate hosts or arthropod vectors with different BTV strains can lead to the exchange of genetic information and the creation of reassortant viruses (Oberst *et al.*, 1987; Samal *et al.*, 1987a; Samal *et al.*, 1987b; Stott *et al.*, 1987; Ramig *et al.*, 1989). The combined length of all segments is approximately 19,200 base pairs, but varies with serotype. Their complete nucleotide sequence had first been determined for BTV-10 (Fukusho *et al.*, 1989). All segments of all known serotypes share short conserved ends (5'-GUUAAA…UUAC-3') (Mertens and Sangar, 1985;

reviewed by Alpar *et al.*, 2009). The genome encodes seven structural and four non-structural proteins (see table 1). Each segment contains one open reading frame flanked by non-coding regions. The open reading frame on segment 10 encodes two proteins by alternate translation initiation (Lee and Roy, 1987). In many strains of BTV this is also the case for segment 9 (Wade-Evans *et al.*, 1992; Maan *et al.*, 2010).

Seg-ment	Size (nt)	Encoded protein	Location (number of copies per virion), proposed function	Protein size (weight)
1	3954	VP1	Within the core (12), RNA-dependent RNA polymerase	1302 aa (150 kDa)
2	2926	VP2	Outer capsid (180), type-specific structural protein	961 aa (111 kDa)
3	2772	VP3	Inner (sub-core) capsid (120), scaffold for VP7 layer	901 aa (103 kDa)
4	2011	VP4	Within the core (24), RNA capping enzyme	644 aa (75 kDa)
5	1770	NS1	Non-structural protein (0), forms tubules of unknown function in host cells	552 aa (64 kDa)
6	1639	VP5	Outer capsid (360), structural protein, co-determinant of virus serotype	526 aa (59 kDa)
7	1156	VP7	Core capsid (780), group-specific structural protein	349 aa (39 kDa)
8	1123	NS2	Non-structural phosphoprotein (0), forms viral inclusion bodies in host cells	354 aa (41 kDa)
9	1046	VP6	Within the core (72), RNA helicase	329 aa (36 kDa)
10	822	NS3	Non-structural glycoprotein (0), membrane protein, aids virus release from host cells	229 aa (26 kDa)
		NS3A	Expressed by alternate translation initiation	216 aa (24 kDa)

Table 1: Bluetongue virus genome segments and proteins. Size (amino acids, aa) and weight (Dalton, Da) data are for the European reference isolate of BTV-8 (Roy, 1992; Grimes *et al.*, 1998; Stuart *et al.*, 1998; Mertens *et al.*, 2004b; Maan *et al.*, 2008; Noad and Roy, 2009)

The outer layer of BTV particles consists of 180 copies of virion protein 2 (VP2) arranged in sixty triskelia (three-fold interlocked spirals), interspersed with 120 globular trimers of virion protein 5 (VP5) (Hewat *et al.*, 1992; Nason *et al.*, 2004). VP2 shows the greatest variability of all BTV proteins (Roy, 1992). It mediates hemagglutination as well as receptor binding during the initiation of infection (Hübschle, 1980; Hassan and Roy, 1999) and contains most of the epitopes that interact with neutralizing antibodies, making it the

main determinant of virus serotype (Huismans and Erasmus, 1981; Appleton and Letchworth, 1983; Kahlon *et al.*, 1983; Mertens *et al.*, 1989).

VP5 is thought to influence the highly conformation-dependent VP2 protein by their close interaction in the outer capsid layer (White and Eaton, 1990), but may also harbor some neutralization sites itself (DeMaula *et al.*, 2000). It is involved in membrane penetration leading to the release of viral particles from endosomes into the cytoplasm, and can also act as a membrane fusion protein *in vitro* (Hassan *et al.*, 2001; Forzan *et al.*, 2004). Before the virus particle actually enters the cytoplasm of an infected cell, the outer capsid layer is lost (Mertens *et al.*, 2004a) (see figure 1).

The remaining core capsid consists of 260 virion protein 7 (VP7) trimers supported by an inner sub-core layer made up of 12 decamers of VP3 (Stuart *et al.*, 1998). The VP7 trimers are easily visible by electron microscopy, and had been described as capsomers arranged in ring-like structures (Els and Verwoerd, 1969), a feature alluded to in the genus name (Latin *orbis, -is* m.: a circle or ring) (Roy, 2007). Together, VP7 and VP3 form the core particle with a diameter of 73 nm (Grimes *et al.*, 1998). The protein sequences of VP3, and to a lesser extent VP7, are highly conserved across the BTV serogroup (Huismans and Erasmus, 1981) and VP7 is the immunodominant group-specific antigen. BTV shares some 60% of VP7 amino acids with EHDV, and 45% with AHSV (Iwata *et al.*, 1992).

Along the five-fold symmetrical axes of the particle, the VP3 decamers leave twelve small pores in their centers. At the pores, the minor structural proteins VP1, VP4 and VP6 form transcription complexes that are closely associated with the genetic material in the central space of the core (Gouet *et al.*, 1999; reviewed by Mertens *et al.*, 2009b). Each complex is made up of one copy of VP1, a VP4 dimer and a VP6 hexamer (Stuart *et al.*, 1998). The available structural and biochemical data are consistent with the assumption that each genome segment is associated with a transcription complex to allow for their independent simultaneous transcription (Nason *et al.*, 2004).

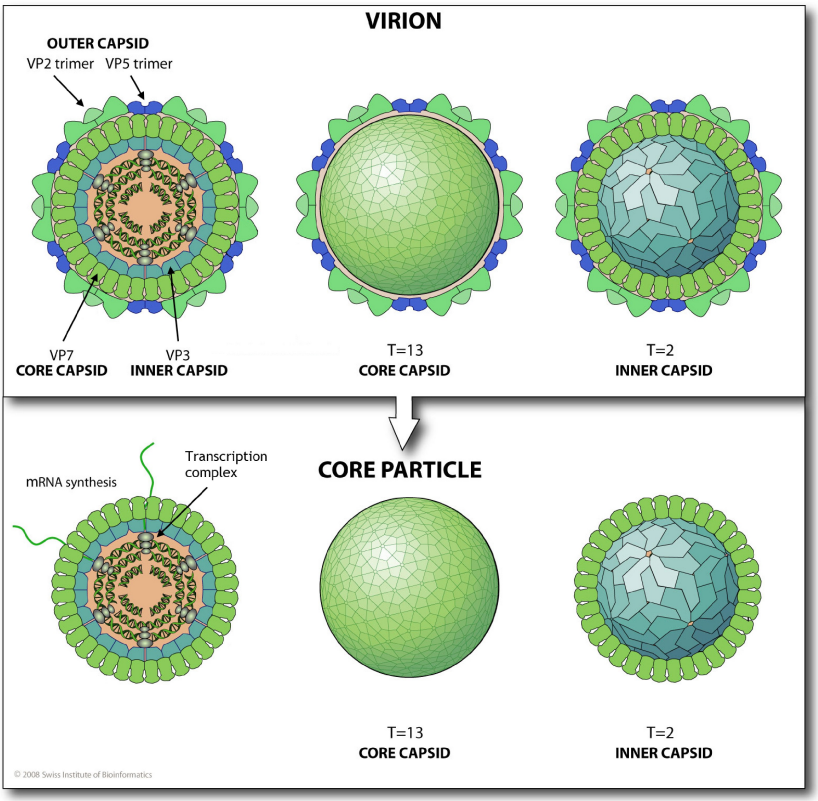

Figure 1: Overview of key structural features of BTV[1].

2.2 History, global distribution and economic impact

Historically, BTV was thought to be confined to southern Africa, where it has probably been endemic in wild ruminants from antiquity. The disease was recognized in the late 18th century, when fine-wooled sheep breeds were introduced to the Cape Colony (Verwoerd and Erasmus, 2004). The first detailed scientific studies date from the beginning of the 20th century. Initially, they referred to the disease as "malarial catarrhal fever", since Hutcheon (1902) had assumed its agent to be an insect-transmitted plasmodium. Spreull (1905)

[1] Source: ViralZone, http://www.expasy.org/viralzone, Swiss Institute of Bioinformatics. Used with permission.

credited Robertson and Theiler with the discovery of the viral nature of the disease, and suggested that the common name “bluetongue” (from the Afrikaans “bloutong”, used by Boer farmers to describe the distinctive cyanotic tongue of severely affected sheep) should be used instead (Gorman, 1990).
The plurality of antigenetically different strains of variable virulence and the implications for immunization against BTV were first described in the 1940s (Neitz, 1948). Eventually, the BTV serogroup was classified into serotypes based on neutralization. While Howell (1960) had defined 16 serotypes, this number subsequently increased to 24 (see review by Gorman, 1990, for the corresponding reference strains). By definition, serotypes are distinct antigenic groups of bluetongue viruses, but there is a varying degree of cross-reactivity in *in vitro* tests as well as cross-protection *in vivo* (Erasmus, 1990) (see figure 2). BTV types that cross-react serologically were also found to have greater similarity in deduced VP2 amino acid sequences (Maan *et al.*, 2007).

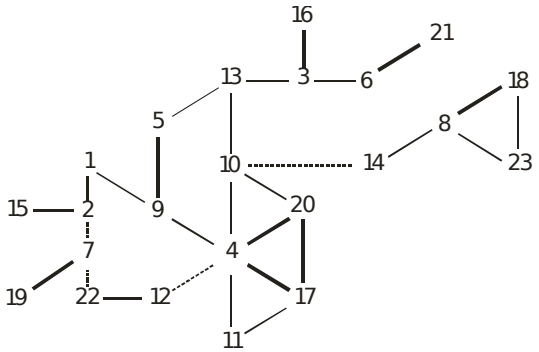

Figure 2: Map of serological relationships between BTV serotypes (Erasmus, 1990), showing BTV-4 as a hypothetical “ancestral serotype”.

An outbreak in Cyprus in 1943 (Gambles, 1949) was the first confirmed occurrence of BTV outside of Africa, followed by reports from the Eastern Mediterranean region (Komarov and Goldsmit, 1951) and the Indian subcontinent (Sarwar, 1962; Sapre, 1964). In the 1950s BTV was confirmed in sheep in California (Hardy and Price, 1952; McKercher *et al.*, 1953). Later genetic analyses, however, indicated that several BTV serotypes share a long evolutionary history in North America (Heidner *et al.*, 1991). In Canada, BTV (serotype 11) has only been detected in the Okanagan Valley of British Columbia (Dulac *et al.*, 1992). Recent warming trends have a favorable influence on indigenous vectors, however, and may allow incursions and the

eventual establishment of BTV in other parts of Canada (Weaver and Reisen, 2010).

BTV has been isolated from both arthropods and vertebrates on all continents except Antarctica. It regularly occurs throughout much of the world between latitudes of approximately 40° to 50° N and 35° S, where it is considered enzootic. Recently, however, BTV has spread far beyond the upper limits of this traditional range into areas of Europe where it had never been reported. Similar detections of previously exotic serotypes have been reported from the southeastern United States, Israel and northern Australia (reviewed by MacLachlan, 2010).

Strains of BTV in different regions of the world are distinct and often exist in stable ecosystems (Gould and Pritchard, 1990). Isolates of the same serotype with different geographical origins (so-called topotypes) can be distinguished by sequence variations in genome segment 2 coding for the outer capsid protein VP2 (Maan *et al.*, 2004; Mertens *et al.*, 2007). Geographic separation allows the acquisition of unique point mutations (Maan *et al.*, 2009). An analysis of full-length segment 2 sequences of all 24 serotypes found nine evolutionary branching points, which correlate with ten nucleotypes defined by <35% difference in nucleotide sequences (Maan *et al.*, 2007). Globally, a distinction is made between virus isolates belonging to the "western" (Africa and the Americas) or "eastern" (Eurasia and Oceania) lineages, respectively (Balasuriya *et al.*, 2008; reviewed by Mertens *et al.*, 2009a). The level of divergence between those lineages indicates long physical separation (Maan *et al.*, 2009).

Not all gene segments of BTV can be used to clearly topotype strains of BTV, however (Bonneau *et al.*, 2000). Segments 7 and 10 show variations that do not simply reflect the geographic origins of the virus isolate (Pierce *et al.*, 1998; Maan *et al.*, 2008). Individual genes evolve independently, in response to the selective pressures encountered within their respective insect and mammalian hosts. Genetic drift during alternating passages in insects and mammals generates quasispecies populations and novel genotypes are fixed by founder effects when unique viral variants are randomly ingested by feeding vectors (Bonneau *et al.*, 2001; reviewed by Bonneau and MacLachlan, 2004).

A major epizootic on the Iberian Peninsula caused by BTV-10 was the first documented occurrence of BTV in Europe outside of Cyprus, starting in 1956 (Manso-Ribeiro *et al.*, 1957; Lopez and Botija, 1958). Within four years, its eradication was achieved by a massive campaign of quarantine, slaughter and vaccination (Gorman, 1990). Bluetongue was then generally considered exotic to Europe until 1998, when BTV-9 was detected on several Greek islands close to the Turkish mainland. This and other serotypes subsequently spread northwards and westwards on the Aegean islands. Meanwhile, BTV-1 had been introduced from Northern Africa to the Iberian Peninsula and Italy. While the

northern movement of BTV-2, -4, -9 and -16, present in the Mediterranean Basin, is apparently restricted, the area affected by BTV-1 has recently expanded across the Iberian peninsula and France (reviewed by MacLachlan, 2010). Bluetongue, originally a tropical and subtropical disease, has regularly occurred in Europe during the last decade (Wilson *et al.*, 2008) (see table 2).

In August 2006, BTV-8 was first detected in the Netherlands (Enserink, 2006). Retrospective studies suggest that the original introduction of BTV-8 had probably occurred during the spring of 2006 near a nature reserve in Eastern Belgium where large numbers of migratory birds congregate (Saegerman *et al.*, 2010b), but many other possible routes of introduction have been proposed (Mintiens *et al.*, 2008). By the end of 2006, cases had also been found in Belgium, France, Luxembourg and Germany (Saegerman *et al.*, 2008), where the first case had been confirmed on August 21, 2006 (Anonymous, 2006). Reports of new cases ceased by January 2007. The virus did not die out at the end of the vector season, however, and reemerged only a short time later (Hoffmann *et al.*, 2008).

Possible explanations for its successful overwintering are listed by Wilson *et al.* (2008), persistence in the vector or host populations being the most prominent. Most species of *Culicoides* at northern latitudes survive the winter as larvae. Experiments designed to look for vertical (transovarial) transmission of BTV in *Culicoides* have consistently reported negative results (reviewed by Wilson *et al.*, 2008). In mild winters, however, it is possible that a small fraction of the infected adult *Culicoides* population might survive long enough to bridge the gap between transmission seasons. If adult midges enter buildings when outdoor temperatures begin to drop, they might experience conditions that are sufficiently warm for blood-feeding year-round (Wilson *et al.*, 2008; Zimmer *et al.*, 2010). This could explain the very early reemergence of BTV-8 in Germany in February 2008 (Hoffmann *et al.*, 2008).

BTV could also covertly persist in the ruminant population. Transmission across the placenta to the fetus has been repeatedly demonstrated for European BTV-8 (De Clercq *et al.*, 2008; Menzies *et al.*, 2008; Backx *et al.*, 2009; Darpel *et al.*, 2009b; Worwa *et al.*, 2009), while evidence for latent infection of ruminants is inconsistent (reviewed by Wilson *et al.*, 2008). BTV does not cause persistent infections, but prolonged viremia in excess of two months has been reported in cattle (Singer *et al.*, 2001). This could permit the virus to overcome short periods of vector absence. Rather than a single overwintering mechanism, there likely is a toolbox of possible mechanisms that interact with and complement one another (Wilson *et al.*, 2008).

Sero-type	First isolate*	Affected regions or countries	Immediate origin	Status
1	2001	Greece	Turkey (suspected) (eastern lineage)	Ongoing (Islands)
	2007	Iberian Peninsula, France, Sardinia	Algeria, Morocco (western lineage)	Ongoing
2	2001	Corsica, Italy, Balearic Islands	Tunisia	Ongoing (Italy)
	2003	Italy	South African (SA) vaccine strain from 2002 campaign	
4	1999	Greece and the Balkans	Related to earlier isolates from Cyprus (1969) and Turkey (1978)	Probably resolved
	2003	Balearic Islands, Italy, Iberian Peninsula	Tunisia (suspected), distinct from strains in the eastern Mediterranean	Ongoing (Italy)
6	2008	Netherlands, Germany (Lower Saxony)	Unknown (SA vaccine strain)	Probably resolved
8	(2006)	Bulgaria	Unknown	Probably resolved
	2006	Europe	Unknown (western lineage)	Ongoing
9	1998	Greece and the Balkans, Bulgaria, Italy	Unknown (eastern lineage)	Ongoing
	2003	Italy	SA vaccine strain from 2002 campaign (western lineage)	
11	(2008)	Belgium	Unknown (South African vaccine strain)	Probably resolved
16	1999	Greece, Cyprus	Unknown (eastern lineage)	Ongoing
	2002	Italy	SA vaccine strain reassortant from Israel (suspected)	
	2004	Croatia, Sardinia	SA vaccine strain from Italian campaign	
TOV	(2008)	Switzerland, Italy, Germany	Unknown	Unknown

Table 2: Bluetongue viruses recently occurring in Europe and their suspected origins. *Parentheses indicate that no isolate was made, but the virus was detected by other means. (Maan *et al.*, 2004; Ferrari *et al.*, 2005; Mertens and Attoui, 2007; Batten *et al.*, 2008b; Hofmann *et al.*, 2008; Saegerman *et al.*, 2008; Anonymous, 2009d; e; h; Listes *et al.*, 2009; Hofmann *et al.*, 2010)

From 2007 onwards, BTV-8 has spread as far as Spain, Norway and Hungary. From August 2006 to October 2009, veterinary authorities in Germany registered BTV-8 in 26,950 animal holdings (Gethmann *et al.*, 2010). After vaccination was introduced in 2008, the number of new cases fell dramatically (data for Germany are presented by Conraths *et al.*, 2009), but the virus has not been eradicated (Gethmann *et al.*, 2010).

In early 2008, "Toggenburg orbivirus" (TOV), a novel orbivirus isolate that might prove to be a 25th BTV serotype, was found in goats in Switzerland (Hofmann *et al.*, 2008). Later that year, BTV-6 and -11 were detected in the Netherlands, Germany and Belgium, respectively. They did not spread to any meaningful extent, and the strains were found to be very closely related to modified-live virus (MLV) vaccines regularly used in South Africa (De Clercq *et al.*, 2009; Eschbaumer *et al.*, 2009b; reviewed by Hateley, 2009). As for BTV-8, the method of introduction of those strains is still unclear (MacLachlan and Guthrie, 2010). The increasing global trade not only in livestock has probably increased the frequency of introduction of exotic viruses to new ecosystems, while climate change in Europe may explain the increasing tendency of those strains to persist and spread there (Wilson *et al.*, 2008).

Bluetongue has repeatedly caused heavy losses particularly in naïve populations of susceptible breeds in non-endemic areas (reviewed by Alpar *et al.*, 2009). The number of sheep that died or were culled during the repeated incursions of several serotypes into the Mediterranean Basin between 1998 and 2005 is estimated at over one million (Purse *et al.*, 2005), a death toll never seen in areas where BTV is enzootic. In the 1990s, annual losses to BT were estimated at US$3 billion worldwide (Tabachnick *et al.*, 1996).

The BTV-8 epizootic in Europe is believed to have caused greater economic damage than any previous outbreak involving a single serotype (Wilson and Mellor, 2008). For 2006 and 2007, the total costs for the Netherlands were estimated at €200 million (Velthuis *et al.*, 2010). In 2007, over €20 million were paid in direct compensation for bluetongue-related animal deaths in Germany (Gethmann *et al.*, 2010), a figure that does not include losses due to decreased production, trade restrictions or costs for treatment and diagnostics. Figure 3 gives an overview of zones in the EU where movement restrictions due to BTV currently apply. These zones can only be lifted after two years of confirmed absence of BTV circulation in an area (European Commission, 2007).

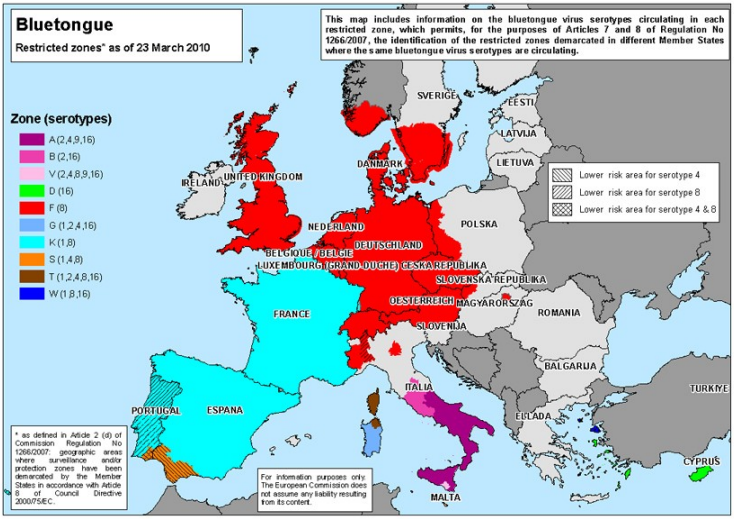

Figure 3: Map of BTV restricted zones in the EU (European Commission, 2010).

2.3 Vectors and transmission

The global distribution of BTV coincides with its main vector, *Culicoides* spp. midges (*Diptera*, *Ceratopogonidae*) (Mellor, 1990), which are associated with the transmission of several viruses, protozoa and filarial nematodes (Meiswinkel *et al.*, 2004). Their role in BTV transmission had first been shown for *C. imicola* Kieffer (Du Toit, 1944). Since then, only about thirty of more than one thousand species in the *Culicoides* genus have been found to be potential vectors of BTV. But even for innately competent vectors, there are important behavioral aspects to consider, such as host preference and seasonal and circadian activity (Kampen, 2009). What is more, within a competent *Culicoides* species, not all female midges are susceptible to BTV infection or capable of transmitting the virus when infected. The proportion of these so-called refractory midges in a population is variable (Mellor, 1990).
Other arthropods can possibly spread the virus by purely mechanical transmission (Luedke *et al.*, 1965), and European BTV-8 was shown to infect ticks (Bouwknegt *et al.*, 2010). Modes of transmission without the involvement of arthropods have been suggested, including iatrogenic transmission by humans. Their epidemiological relevance, however, is mostly negligible. The

geographical distribution and seasonal incidence of BTV is largely determined by the presence of suitable vectors (Venter *et al.*, 2009), with the *Culicoides* species or species complex predominantly responsible for the transmission of BTV differing between regions of the world (MacLachlan *et al.*, 2009). *C. imicola* is not involved in the BTV-8 epizootic in Central Europe (Meiswinkel *et al.*, 2008), and the *C. obsoletus* species complex is assumed to be predominantly responsible for BTV transmission in Germany (Mehlhorn *et al.*, 2007; Hoffmann *et al.*, 2009a).

BTV seasonality is influenced by ecological factors such as rainfall, temperature and humidity (Erasmus, 1990). During their active period, adult female midges feed on blood to obtain protein for the production of eggs. If their host is infected with BTV, virus particles from the blood meal replicate in cells of the mid-gut epithelium of the midge. Progeny virus is released into the hemolymph and infects secondary target organs. Transmission during subsequent feedings on vertebrate hosts can take place only after virus replication in the salivary glands of the midge. At an ambient temperature of 25 °C, this takes ten to fifteen days after initial infection of the midge. Once infected with BTV, midges remain so for life, with no apparent disadvantage to the insect (Mellor, 1990). There is molecular evidence for vertical transmission in midges (White *et al.*, 2005), but so far virus has never been isolated from larvae.

In any case, there are means of BTV spread that do no rely on arthropod vectors at all. Semen from infected bulls collected during the viremic phase may be infective, and cows reportedly have been infected by such semen (Bowen *et al.*, 1985). There is limited evidence for horizontal transmission between infected and not infected animals in close-contact conditions (Jochim *et al.*, 1965; López-Olvera *et al.*, 2010), and calves can be experimentally infected by contaminated colostrum (Backx *et al.*, 2009). Vertical transmission along with teratogenic potential has been described for modified-live virus vaccines for bluetongue, but is highly unusual for field strains of BTV. The passage of BTV strains in heterologous culture systems can confer undesirable properties: The most prominent feature of laboratory-adapted virus is the ability to cross the placenta, which can lead to fetal malformation and abortion. Teratogenic effects of vaccination with modified-live virus vaccines were first described more than fifty years ago (Shultz and Delay, 1955). These effects are now well recognized and vaccination of pregnant ewes with live vaccines is discouraged (reviewed by Osburn, 1994).

On the other hand, fetal infection following natural exposure of ruminants to wild-type viruses is rare. There have been reports of abortion after infection of sheep with pathogenic strains, but this was considered a consequence of high fever in the ewe. In regions where live vaccines have never been used (such as Australia), there was no evidence of virus crossing the placenta (Kirkland and

Hawkes, 2004). The recently discovered TOV reportedly caused transplacental infection in goats (Chaignat *et al.*, 2009).
For the European strain of BTV-8, however, transplacental transmission and infection of cattle by direct contact with infected placental tissue was first reported in February 2008 (Menzies *et al.*, 2008). Zoo carnivores had been fatally infected by the ingestion of aborted fetal ruminants (Jauniaux *et al.*, 2008). Evidence for transplacental transmission in cattle and sheep was collected in field studies (Darpel *et al.*, 2009b; Saegerman *et al.*, 2010a; Santman-Berends *et al.*, 2010), corroborating experimental findings (Backx *et al.*, 2009; Worwa *et al.*, 2009). This suggests that the European field strain of BTV-8 might have picked up properties of *in vitro* passaged bluetongue viruses at some point in the past (MacLachlan and Osburn, 2008; MacLachlan, 2009).
Even if vertical transmission is only secondary in BTV epidemiology, it can become increasingly important in periods or areas with little or no *Culicoides* activity (Gibbs *et al.*, 1979; Santman-Berends *et al.*, 2010). Up to now, live virus has been successfully isolated only from transplacentally infected calves that received either no maternal colostrum or only pooled colostrum (reviewed by Darpel *et al.*, 2009b). The failure to reisolate virus *in vitro*, however, does not necessarily preclude infection of midges feeding off these calves (Santman-Berends *et al.*, 2010). In any case, the combined serological and real-time RT-PCR results of pregnant dams give no clear indication of the infection status of the unborn except in the case of a double negative result (De Clercq *et al.*, 2008), which is an important consideration for animal trade.

2.4 Host range

The host range of BTV is very broad, from sheep, cattle and goats to deer, mouflon, antelopes and various other even-toed ungulates, including camelids. The outcome of infection ranges from fatal to subclinical, with the latter being predominant. Since BTV infection of cattle usually goes unnoticed and results in prolonged viremia, they are considered important reservoir hosts. Historically, the primary vertebrate host species probably were African antelopes (Erasmus, 1990).
Although ruminants and camelids are the most important hosts of BTV, other species can be affected. In the 1990s, there were several fatalities in pregnant dogs that had been accidentally infected by a canine vaccine contaminated with BTV-11 (Evermann *et al.*, 1994). This was later confirmed by corroborative experiments (Brown *et al.*, 1996). Following this incident, an extensive field survey detected antibodies to BTV in African carnivores, but only in species

that prey on wild ruminants (Alexander *et al.*, 1994). The European strain of BTV-8 was reported to have killed European lynx that had been infected by the ingestion of aborted fetal ruminants (Jauniaux *et al.*, 2008). African Horse Sickness virus (AHSV), a closely related orbivirus species, reportedly infected carnivores that were fed horse meat (Van Rensberg *et al.*, 1981).
Intracranial infection of newborn mice has been used in BTV research and vaccine production for over fifty years (Van Den Ende *et al.*, 1954; Svehag, 1962; Franchi *et al.*, 2008). More recently, interferon receptor-deficient adult mice were experimentally shown to be susceptible to fatal BTV infection (Calvo-Pinilla *et al.*, 2009a).

2.5 Virus replication

In nature, BTV is maintained by an endless series of alternating cycles of replication in *Culicoides* midges and various mammalian species (Mellor, 1990). Accordingly, the virus has evolved to optimize its transmission efficiency within the context of the vector/host relationship. Its replication mechanisms and strategies within the mammalian host have consequences for transmission to the insect vector and vice versa (Darpel *et al.*, 2009a).
A single bite from a transmission-competent infected midge will reliably infect susceptible sheep, while experimental infection by needle inoculation requires much higher amounts of virus. It is presently unclear why transmission from the vector to the host is more efficient, but it has been suggested that immunomodulatory proteins in the saliva of the vector may play an important role (reviewed by Darpel *et al.*, 2009a). Relatedly, bites by *Culicoides* spp. can lead to hypersensitivity reactions in many species, including sheep (Connan and Lloyd, 1988) and are the cause of so-called “sweet itch” in horses (Mellor and McCraig, 1974).
After inoculation by the midge, BTV is transported to regional lymph nodes by migrating dendritic cells (Hemati *et al.*, 2009). After initial replication in the lymph node (Pini, 1976; Barratt-Boyes *et al.*, 1995), virus progeny is released into the blood stream and large amounts of virus are then produced in peripheral blood mononuclear cells (PBMC) and endothelia (Pini, 1976; Mahrt and Osburn, 1986; Barratt-Boyes and MacLachlan, 1994) (see also figure 4). High levels of early replication have recently also been reported in skin. Historically, this might have been underestimated, because virus isolation is hindered by the fibrous and compact nature of skin tissue. The skin may be both an important site for replication and a source of virus for blood-feeding vectors (reviewed by Darpel *et al.*, 2009a).

On a cellular level, replication occurs principally in mononuclear phagocytic cells, endothelial cells and lymphocytes undergoing blastogenesis (Mahrt and Osburn, 1986; MacLachlan *et al.*, 1990; Barratt-Boyes *et al.*, 1992; Ellis *et al.*, 1993; reviewed by MacLachlan *et al.*, 2009), whereas the interaction with erythrocytes and unstimulated lymphocytes does not proceed beyond attachment of the virus to the cell membrane (Brewer and MacLachlan, 1994).

Viremia in ruminants can persist for extended periods of time, even in the presence of high titers of neutralizing antibody (Luedke, 1969; Luedke *et al.*, 1969; reviewed by Singer *et al.*, 2001). It has been shown that this apparent immune evasion is not due to antigenic drift (Heidner *et al.*, 1988). While infectious virus has been recovered from blood as late as two months after experimental infection of cattle, circulating viral RNA can be detected even longer (Katz *et al.*, 1994). This is achieved by passive association with red blood cells (MacLachlan *et al.*, 1994; Brodie *et al.*, 1998). Accordingly, the duration of detection correlates with the lifespan of erythrocytes (Brewer and MacLachlan, 1994). Bluetongue virions bind to glycophorins on the surface of ruminant erythrocytes and escape neutralization in invaginations of their membrane, thus extending the period of possible infection of blood-sucking midges (Eaton and Crameri, 1989). Although viremia is prolonged in BTV-infected cattle, it is not persistent and recovered animals are immune to reinfection with the homologous serotype of BTV (MacLachlan *et al.*, 1990). One *in vitro* experiment suggested a potential for persistent BTV infection of lymphocytes (Takamatsu *et al.*, 2003), but this could not be confirmed in naturally infected animals (Melville *et al.*, 2004).

In vitro, BTV can also replicate in a wide range of cells, from mammalian epithelia and endothelia to insect cells originating from midges, mosquitoes and flies (Ross-Smith *et al.*, 2009). In cultured cells, field isolates often replicate poorly at first, but adapt readily (Roy, 2007). BTV core particles, which have been stripped of their outer capsid layer, have a much reduced infectivity for mammalian cells, but can still infect insect cells (Mertens *et al.*, 1996), indicating a distinct cell attachment and penetration capability of VP7 (Xu *et al.*, 1997).

Insect-derived cell lines (including *C. sonorensis* [= *C. variipennis*] embryonic cells) can be productively infected without an increase in cell death. On the other hand, BTV was shown to induce apoptosis and cytopathic effect (necrosis) in several mammalian cell lines (Mortola *et al.*, 2004), with possible implications for pathogenesis in the vertebrate host (DeMaula *et al.*, 2001; DeMaula *et al.*, 2002a). In baby hamster kidney (BHK) cells, host cell translation is reportedly shut off within 10 hours of infection (Mertens *et al.*, 1984). BSR cells (a BHK clone) were shown to remain fully viable for the first

day after infection, but viability decreased more than 10-fold within the next two days, even at low initial multiplicities of infection (Li *et al.*, 2009).

On a cellular level, the outcome of infection is influenced by the mechanism of virus release (reviewed by Ross-Smith *et al.*, 2009). Unlike the budding of progeny virions from insect cells, virus release from mammalian cells causes damage to the cell membrane, possibly culminating in cell death (Owens *et al.*, 2004). The extent of damage, however, varies in the course of infection. A considerable number of infectious progeny is released before the onset of cytopathic effect (Noad and Roy, 2009), suggesting a different, less harmful mode of virus egress in the early stages (Hyatt *et al.*, 1989; Eaton *et al.*, 1990). Production of mature particles is exponential between 8 and 24 hours post infection (reviewed by Schwartz-Cornil *et al.*, 2008).

In order to infect host cells, viruses must transport their genomic material across a lipid bilayer. For mammalian cells, the BTV outer capsid protein VP2 mediates cell attachment and endocytosis in clathrin-coated vesicles (Forzan *et al.*, 2007). The cellular receptor for BTV is assumed to be a glycoprotein (Eaton and Crameri, 1989), and purified VP2 has also been shown to have strong affinity for glycophorin A (CD235a), a sialoglycoprotein component of erythrocytes (Hassan and Roy, 1999) that has also been identified as a cellular receptor for several other viruses (see for example Wybenga *et al.*, 1996; Thacker and Johnson, 1998; Sanchez *et al.*, 2004). A sialic-acid binding domain on BTV VP2 has recently been identified by cryo-electron microscopy (Zhang *et al.*, 2010).

Within the endosome, VP2 dissociates from the outer capsid layer, and the low pH activates VP5-mediated membrane fusion and permeabilization (Hassan *et al.*, 2001; Forzan *et al.*, 2004). Zhang *et al.* (2010) recently proposed a perforation mechanism involving 12 amphipathic helical regions on the external surface of VP5.

After the outer capsid layer is lost, virus cores are released into the cytoplasm. The core particle does not disassemble further. In the cytoplasm, it is transcriptionally active, synthesizing and extruding multiple capped but not polyadenylated message-sense RNA copies of each double-stranded RNA segment of the viral genome (Verwoerd and Huismans, 1972; Mertens *et al.*, 2004a). This viral mRNA is produced by transcription complexes formed by VP1, VP4 and VP6 at the core particle pores (Gouet *et al.*, 1999; Noad and Roy, 2009). Individually, VP4 has been shown to be the mRNA capping enzyme (Martinez-Costas *et al.*, 1998), while VP1 is the RNA-dependent RNA polymerase (Urakawa *et al.*, 1989). The third minor core protein, VP6, has helicase activity (Stäuber *et al.*, 1997). Together, they unwind double-stranded viral RNA, synthesize single-stranded RNA of both polarities and modify the 5’

termini of newly synthesized viral mRNA molecules for efficient translation by the host cell (Roy, 2007).

In cell culture, the first BTV-specific proteins (NS1 in particular) are detectable within two to four hours after infection (Huismans and Van Dijk, 1990). Core structural proteins produced by the host-cell translation machinery are assembled in viral inclusion bodies (virus assembly factories) (Roy, 2007) whose major component is the NS2 phosphoprotein (Eaton *et al.*, 1990). Inclusion bodies in infected cells are aggregated with BTV RNA, and NS2 also appears to be involved in the selective packaging of genome segments into progeny particles (Lymperopoulos *et al.*, 2006), while the polymerase enzyme VP1 is not substrate-specific (Lymperopoulos *et al.*, 2003). Once formed, new cores are transcriptionally active until the outer capsid proteins VP2 and VP5 are added to the virus particle.

The subsequent release of progeny virions varies between host cell types. Virus progeny are released both as enveloped particles by budding through the plasma membrane (although the envelope is only short-lived) and as non-enveloped particles by extrusion through the membrane (Hyatt *et al.*, 1989), processes that involve NS1 and NS3 (Beaton *et al.*, 2002; Owens *et al.*, 2004). While NS1 and NS2 are always synthesized abundantly (the former makes up 25% of total BTV proteins in an infected cell) (Roy, 2007), the expression levels of the membrane proteins NS3 and NS3A (which lacks the 13 N-terminal amino acids of NS3) depend on the host cell species. They are generally high in insect cells, and low in cells of vertebrate origin (French *et al.*, 1989; Guirakhoo *et al.*, 1995). The extent of their production appears to be positively correlated with the efficiency of virus release. NS3 interacts with TSG101, a cellular protein implicated in the trafficking and release of enveloped viruses, whose knockdown with small interfering RNA was shown to inhibit BTV release from mammalian cells (Wirblich *et al.*, 2006). The precise function of the perinuclear cytoplasmic tubules formed by NS1 multimers, a striking feature of BTV infection, presently remains unclear, while experiments *in vitro* and *in vivo* have implicated the protein as a major determinant of pathogenicity in the vertebrate host (reviewed by Kirkland and Hawkes, 2004; Owens *et al.*, 2004). Throughout the viral replication process, the core shell serves as a physical barrier between the cell cytoplasm and the double-stranded viral RNA (Mertens *et al.*, 2004a), hiding the latter from the defense mechanisms of the host cell (Jacobs and Langland, 1996). Accordingly, progeny virus double-stranded RNA is synthesized to viral mRNA templates within newly formed cores and only the 5’ terminus of the coding strand of each segment is capped (Roy, 2007).

2.6 Pathogenesis and clinical disease

The clinical manifestation of BTV infection varies considerably between species, breeds and virus strains (reviewed by MacLachlan *et al.*, 2009). Serotype does not determine virulence, there are both highly virulent and benign strains within the same serotype (Kirkland and Hawkes, 2004; MacLachlan *et al.*, 2009). Although BTV can infect many species of ruminants, clinical signs of disease are generally associated with sheep (Erasmus, 1990). Still, indigenous breeds from regions where BTV is endemic are rarely affected. Some European breeds, on the other hand, appear to be particularly susceptible (Jeggo *et al.*, 1987; Veronesi *et al.*, 2005; Darpel *et al.*, 2007; Worwa *et al.*, 2010). Environmental conditions, particularly exposure to sunlight, reportedly have a marked influence on disease severity (Eisa *et al.*, 1980), with possible implications for animal experiments in high-containment animal housing with artificial lighting (Erasmus, 1990). Immunodeficiency due to concurrent infections can exacerbate the severity of bluetongue disease (Brodie *et al.*, 1998).

Infected cattle usually do not develop overt clinical signs, but the European strain of BTV-8 caused disease in a larger percentage of infected bovines than ever reported before. Field observations from the BTV-8 epizootic found a higher incidence of edema, coronitis, dyspnea and actual “blue tongue” in sheep than in cattle, while cattle more frequently presented crusts and lesions of the nasal mucosa, conjunctivitis and teat lesions (Elbers *et al.*, 2008). The case fatality rate in sheep was found to be at least three-fold higher than in cattle (Conraths *et al.*, 2009). BTV infection of cattle has recently been reviewed extensively (Dal Pozzo *et al.*, 2009), with particular regard to infections with European BTV-8.

In goats, bluetongue disease is rarely seen and when it does occur, it is mostly mild (Backx *et al.*, 2007). Interestingly, the recent detection of TOV, a putative 25th BTV serotype, was made in clinically healthy goats (Hofmann *et al.*, 2008).

Infection of wild ruminants usually is asymptomatic (Rodriguez-Sánchez *et al.*, 2010), but fatalities in mouflon (*Ovis orientalis musimon*) have been reported (Fernández-Pacheco *et al.*, 2008). In 2007, during the BTV-8 epizootic in Europe, seroprevalence in hunted red deer (*Cervus elaphus*) in Belgium was found to be over 40%, with no apparent increase in spontaneous mortality (Linden *et al.*, 2008). Wild ruminants found dead in western Germany were also serologically examined. In the 2007/2008 hunting season, antibodies to BTV were detected in 45% of red deer (91 of 203 animals), but not in roe deer (*Capreolus capreolus*; 499 animals) (Lutz, 2008). In the following season,

prevalence in red deer was lower (7 of 27, 26%), but BTV antibodies were found in 2% of roe deer (3 of 129). In mouflon, prevalence was stable (6 of 45, 13% and 2 of 11, 18%, respectively) (Lutz, 2009).

Experimental infection of red deer with BTV-1 and -8 led to prolonged presence of viral RNA in blood and seroconversion, but no clinical disease was recorded (López-Olvera *et al.*, 2010). Interestingly, North American white-tailed deer (*Odocoileus virginianus*) are highly susceptible to BT (Vosdingh *et al.*, 1968). Expression levels of the Toll-like receptor 3 gene have recently been put forward as a possible explanation for observed differences in disease severity (Vos *et al.*, 2009).

Bluetongue clinical signs in infected mammals can vary widely, from apathy and weight loss to swollen heads, tender feet and death (Wilson *et al.*, 2008), or fever, oronasal ulceration, facial edema, coronitis, muscle necrosis, and pulmonary edema, respectively (MacLachlan *et al.*, 2008). Another peculiar feature of BTV infection of sheep are focal intramural hemorrhages at the base of the pulmonary artery (Roy, 2007).

At the center of BTV pathogenesis lies virus-mediated injury to small blood vessels. Virus replication in endothelial cells causes cell injury and necrosis, potentially leading to hemorrhage, thrombosis and tissue infarction (Mahrt and Osburn, 1986). BTV infection was shown to activate pulmonary endothelial cells and macrophages (DeMaula *et al.*, 2001; DeMaula *et al.*, 2002a; DeMaula *et al.*, 2002b; Drew *et al.*, 2010). Similar to the pathogenesis of viral hemorrhagic fevers like Ebola, host-derived cytokines and other vasoactive substances contribute to the endothelial dysfunction and increased vascular permeability that characterizes fulminant BT (reviewed by MacLachlan *et al.*, 2009).

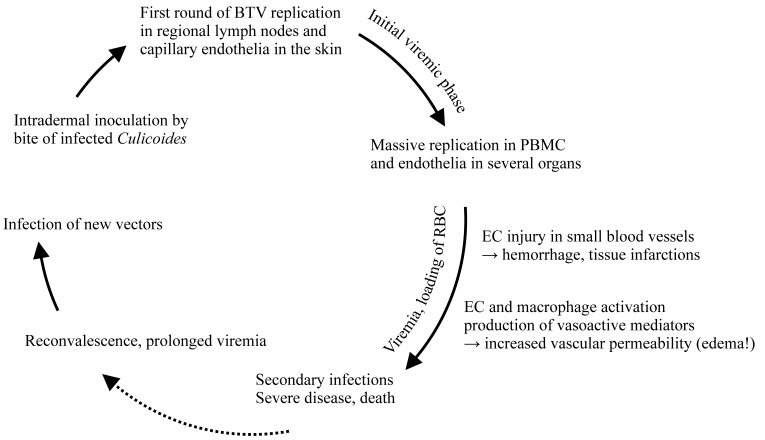

Figure 4: The cycle of BTV infection
(PBMC: peripheral blood mononuclear cells, RBC: red blood cells,
EC: endothelial cells)

2.7 Immune response

The interaction of bluetongue virus with the immune system of the mammalian host has been studied extensively. BTV infection causes panleucopenia with a minimum at 7 to 8 days post infection (Ellis *et al.*, 1990). BTV is also a potent inducer of type I interferon in sheep (Foster *et al.*, 1991), cattle (MacLachlan and Thompson, 1985) and mice (Jameson *et al.*, 1978).
After BTV infection, neutralizing antibodies appear within one or two weeks (Foster *et al.*, 1991) and play a central but not exclusive role in protection. Ruminants that have recovered from BTV infection are immune to reinfection with the same serotype, possibly for life (Ward and Carpenter, 1997). Reconvalescent serum and monoclonal antibodies can passively protect against homotypic BTV infection, as can colostrum-derived maternal antibodies, even from vaccination with inactivated vaccines (Jeggo *et al.*, 1984b; Oura *et al.*, 2010). Infection with a single serotype offers no lasting cross-protection against other serotypes (Jeggo *et al.*, 1986).
Animals experimentally infected with one BTV serotype produce neutralizing antibodies to only that serotype. Serial infections with different BTV serotypes, however, elicit a broad neutralizing antibody response even to serotypes not

encountered previously (Jeggo *et al.*, 1983; Jeggo *et al.*, 1986). After vaccination with outer capsid proteins alone, there is good correlation between neutralizing antibody response and type-specific protection against challenge (Huismans *et al.*, 1987; Roy *et al.*, 1990).

Inactivated whole virus vaccines, on the other hand, can also protect against virulent challenge without inducing any measurable neutralizing antibody response at all (Stott *et al.*, 1985). Similarly, the degree of immunity after BTV infection is not correlated to the level of neutralizing antibodies, even against homologous challenge (Jeggo *et al.*, 1984b), suggesting the involvement of other factors in the outcome of subsequent infections.

BTV-specific cytotoxic (CD8+) T-lymphocytes (CTL) were demonstrated in mice, sheep and cattle (Jeggo and Wardley, 1982; Jeggo *et al.*, 1984a; Ellis *et al.*, 1990). In transfer experiments, recipient sheep were afforded partial protection by B-cell depleted thoracic duct lymphocytes from their reconvalescent monozygotic twins. This partial immunity was not strictly type-specific (Jeggo *et al.*, 1984a; Jochim, 1985). Relatedly, in a vaccination experiment with bluetongue VP7, expressed by a heterologous virus vector, a certain degree of protection was detected in the complete absence of neutralizing antibodies (Wade-Evans *et al.*, 1996).

BTV-specific CTL were found to be mostly serotype cross-reactive in *in vitro* assays (Jeggo *et al.*, 1984a; Takamatsu and Jeggo, 1989). This cross-reactivity, however, seems not to correlate with the relationships between serotypes evident in the cross-reaction of neutralizing antibodies, which could indicate that the cellular and humoral immune responses involve different viral proteins (Takamatsu and Jeggo, 1989). Little is known about the location of antigenic sites for T cells, however. Important variations in the protein targets of CTL exist between individuals and host species (reviewed by Schwartz-Cornil *et al.*, 2008). In mice, CTL predominantly recognize non-structural proteins (Jones *et al.*, 1996), while in sheep, VP2 and NS1 were found to be major immunogens for CTL (Takamatsu *et al.*, 1990; Andrew *et al.*, 1995). Not surprisingly, it was found that heterotypic responses are directed to conserved non-structural proteins rather than to the variable outer capsid.

2.8 Diagnosis and control

Bluetongue is not contagious. Excretions and secretions from infected animals only contain minimal amounts of virus, and susceptible animals generally are refractory to oral infection. Products from infected animals such as meat, milk and wool, are harmless from an epidemiological point of view, even though this does not apply to semen and embryos (reviewed by Erasmus, 1990).
BTV can be introduced into new areas by movement or migration of infected ruminants, and infected midges can be dispersed with livestock, vehicles, trade shipments or on the wind (reviewed by Mintiens *et al.*, 2008). Prevailing winds can carry midges over long distances (Ducheyne *et al.*, 2007; Hendrickx *et al.*, 2008). Wind-borne spread of infected midges across the sea is the suspected mode of introduction of BTV-1 from Northern Africa to the Iberian peninsula and Italy (Wilson and Mellor, 2008). It has even been proposed that serotypes of BTV that occur in Africa can be spread to the Americas by virus-infected insects carried on the trade winds, a theory that is consistent with genetic topotyping studies of global BTV strains (Gibbs and Greiner, 1994; MacLachlan and Guthrie, 2010).
Conventional control measures such as the destruction of infected animals or herds are not effective against BTV. Movement restrictions, however, can help in slowing down the spread of the virus, by preventing the shipment of infected animals over long distances (BVET, 2009). Once BTV has established itself in a region, it may be impossible to eradicate and difficult to control, on account of its wide host range and the probable existence of clinically inconspicuous carrier animals. Likewise, the eradication or even an effective reduction of the vector is currently not possible and ecologically undesirable (Erasmus, 1990). Methods for vector control include insecticides, larvicides and sterilization of males by irradiation (Verwoerd and Erasmus, 2004). Insect-proof housing can prevent vector exposure, but this is not feasible for many sectors of the livestock industry (Orrù *et al.*, 2004). The exposure of sheep to *C. imicola* can reportedly be reduced by keeping cattle in close proximity, because this vector species has a preference for the latter (Nevill, 1978).
After BTV-8 had been introduced to northern Europe in 2006, initial attempts at disease control by animal movement restrictions and insecticides were not successful (Gethmann *et al.*, 2010), even though several insecticides were experimentally shown to be effective against *Culicoides* (reviewed by Schmahl *et al.*, 2009). In the EU, vaccination is considered the principal, and possibly the only, effective veterinary measure in response to bluetongue, accompanied by ancillary measures such as movement restrictions and surveillance (Van

Goethem, 2008). The OIE maintains a list of prescribed BTV tests for international trade (OIE, 2009b).
The reliable, rapid detection and identification of newly introduced BTV strains is required for the implementation of appropriate vaccination strategies (Mertens *et al.*, 2009a). As a counter-example, some 60,000 sheep were vaccinated against BTV-4 in Turkey in 1999, while it was later confirmed that the outbreak in question had been caused by BTV-9 (Maan *et al.*, 2004).

2.8.1 Diagnostic methods and considerations

For a positive diagnosis, BTV antigen, RNA or antibodies must be detected in a sample by virus isolation, molecular or serological methods. Clinical and pathological diagnosis can only be presumptive and requires laboratory confirmation, but it can provide an early indication of infection, particularly in areas where BTV is exotic and not included in routine surveillance (Mertens *et al.*, 2009a). To avoid false-negative results in laboratory diagnosis, serogroup-specific assays for BTV must be able to detect all known BTV serotypes and strains. On the other hand, they must also be able to reliably distinguish these from other viruses, especially those that cause similar clinical signs. Based on clinical symptoms, differential diagnosis in cattle can include foot-and-mouth disease and other vesicular diseases, bovine viral diarrhea, infection with bovine herpesvirus type 1, malignant catarrhal fever, epizootic hemorrhagic disease (caused by a closely related orbivirus not occurring in Europe) and non-infectious conditions like photosensitization or intoxications (Bexiga *et al.*, 2007; Elbers *et al.*, 2008). A wide range of infectious and non-infectious causes of bluetongue-like symptoms in sheep is given by Verwoerd and Erasmus (2004).
Acute BTV infection is characterized by viremia, and virus can be isolated from blood (particularly the cellular fraction) and other tissues by intravascular injection of embryonated chicken eggs or inoculation of permissive cell cultures (Goldsmit and Barzilai, 1968; Bando, 1975; Sawyer and Osburn, 1977; Clavijo *et al.*, 2000). Many mammalian cell lines support BTV replication, but cells of insect origin appear to have a higher sensitivity for native field strains (Mertens *et al.*, 1996). BTV amplification in chicken eggs or cultured cells can then be verified by specific assays. Immune staining using reconvalescent sera or monoclonal antibodies, as well as nucleic acid hybridization techniques, can be used to detect viral proteins in cell culture and tissues (Afshar, 1994).
BTV RNA extracted from a sample can be selectively amplified by reverse transcription polymerase chain reaction (RT-PCR) and detected by agarose gel

electrophoresis or real-time fluorogenic RT-PCR assays. This currently is the most sensitive method of BTV detection (Mertens *et al.*, 2009a). Phylogenetic comparisons of the 24 BTV reference strains show a perfect correlation between sequence variation of the highly variable genome segment 2 (coding for VP2) and serotype (Maan *et al.*, 2007). Conversely, segments 1, 3, 4 and 5 are highly conserved across serotypes. Therefore, depending on primer and, where applicable, fluorogenic probe design, RT-PCR analysis can be used to detect any bluetongue virus, or only certain serotypes or strains (reviewed by Hoffmann *et al.*, 2009b). Group-specific real-time RT-PCR assays targeted at conserved regions on segments 1 and 5 have been validated successfully and are in regular use at many laboratories (Shaw *et al.*, 2007; Toussaint *et al.*, 2007; Batten *et al.*, 2008a). Positive RT-PCR results, however, must be interpreted carefully, since BTV RNA can remain detectable in blood long after the last positive virus isolation (Bonneau *et al.*, 2002; Hoffmann *et al.*, 2009b) (see also figure 5).

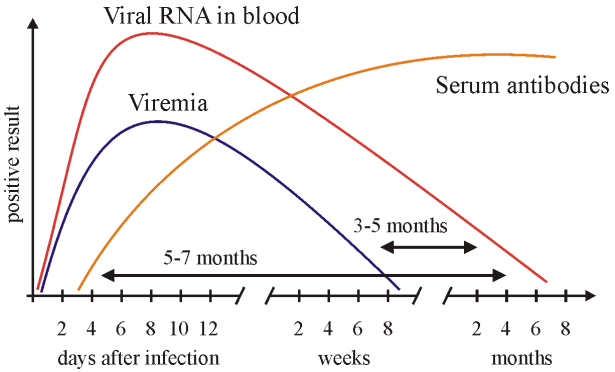

Figure 5: Timeline of BTV diagnostic parameters, showing the long period of concurrent detection of viral RNA and antibodies, as well as the presence of detectable viral RNA long after the end of infectious viremia.

Typing of BTV isolates by molecular assays is considerably faster than virus neutralization tests, and independent of reference virus stocks or sera. PCR primers and probes are readily available from many commercial suppliers. RT-PCR can easily identify and analyze multiple infections with different serotypes and can be used to differentiate between field and vaccine strains, which is difficult or impossible with traditional methods. Amplified cDNA fragments obtained by RT-PCR can be sequenced and compared to published sequences

for confirmation of serotyping and molecular epidemiology studies (Mertens *et al.*, 2009a)

Historically, antibody detection in serum samples relied on complement fixation and agar gel immunodiffusion. Both, however, proved inferior to enzyme-linked immunosorbent assays (ELISA) and were eventually replaced (Hamblin, 2004). Highly sensitive antibody ELISAs can recognize the humoral response to BTV as early as one week after infection (Batten *et al.*, 2008a; Oura *et al.*, 2009). Several different systems are commercially available, mostly using recombinantly produced VP7 protein, which is largely conserved across all serotypes. Indirect ELISAs are available for milk testing (Kramps *et al.*, 2008).

Cell culture-based neutralization tests, first described in the 1950s (Haig *et al.*, 1956), are used to detect serotype-specific antibodies in samples by measuring their neutralizing activity on reference virus. A reverse method, the virus neutralization test, can be used to type virus isolates by incubation with reference sera. Neutralization tests are labor-intensive and require virus stocks and sera which are not commercially available. Therefore, they are not widely used for routine surveillance and typing, but are a useful research tool (Mertens *et al.*, 2009a).

Both ELISA and real-time RT-PCR, on the other hand, are suitable for high-throughput automation and screening of large animal populations. Generally, RT-PCR assays can detect BTV infection earlier than serological assays. In reconvalescent animals, antibodies are detectable for long periods, possibly for life (Mertens *et al.*, 2009a). This is where diagnostic and control strategies for BTV can be at cross purposes, because the reliable differentiation between infected and vaccinated animals is difficult. Theoretically, inactivated vaccines should only elicit antibody responses to structural proteins. The discrimination potential of non-structural protein-based ELISAs has been demonstrated (Anderson *et al.*, 1993; Barros *et al.*, 2009), but this is highly dependent on the purity of the vaccine. The carryover of non-structural proteins from the culture system used to produce the vaccine can result in antibodies to those proteins in vaccinated animals (Alpar *et al.*, 2009). In any case, vaccination campaigns using inactivated whole virus vaccines inevitably compromise VP7-based serological surveillance (Mertens *et al.*, 2009a). Since inactivated vaccines do not contain replicating virus, vaccinated animals usually remain negative in the RT-PCR. For highly sensitive real-time assays, however, this has been disputed (Steinrigl *et al.*, 2009).

For live vaccines, the notable differences in segment 2 sequence even between strains of the same serotype often allow their discrimination from field strains (Mertens *et al.*, 2007), but this is only applicable during the viremic period. Serologically, animals vaccinated with live vaccines cannot be discerned from those that had been infected with field strains of the same serotype.

2.8.2 Vaccination

Generally, protection against a viral disease can be accomplished either by immunization with live but attenuated viruses, inactivated virus particles or sub-particle units. The latter can either be extracted directly from infectious material or produced in a heterologous system by genetic engineering of bacterial, yeast, or other cellular systems into which the gene is introduced. Certain viruses can also be used as vectors for gene expression either in culture or directly in the host (Roy *et al.*, 1990).

Different strategies against bluetongue have been used in the past. At the turn of the 20th century, the viral nature of the etiological agent of BT was discovered in South Africa and first attempts at immunization were made soon after (Theiler, 1908). Initially, lowly virulent BTV strains were utilized. For four decades, the original Theiler strain was maintained solely by passage in sheep, despite evidence that the vaccine was not entirely safe and that the resultant monotypic immunity was inadequate (reviewed by Kirkland and Hawkes, 2004). Eventually, strains that had been attenuated by repeated passages in embryonated chicken eggs were introduced (Alexander *et al.*, 1947).

When cell culture systems for BTV became available, these were used for virus propagation. This process offers the additional benefit of being able to refine the vaccine strain by plaque purification (reviewed by Verwoerd and Erasmus, 2004).

There is a delicate balance between achieving an acceptable reduction in virulence while at the same time maintaining the required level of immunogenicity. Live vaccines have been shown to cause disease in some breeds of sheep (Veronesi *et al.*, 2005; Monaco *et al.*, 2006; Veronesi *et al.*, 2010), can be transmitted by vectors (Ferrari *et al.*, 2005) and have an altered tissue tropism that can lead to teratogenic effects in pregnant animals (Kirkland and Hawkes, 2004) and virus excretion in semen (Kirkland *et al.*, 2004).

In general, replication of the vaccine virus results in the development of a strong immune response. There are numerous concerns over the use of live vaccines, however, especially in an epizootic situation. In general, the use of live vaccines should be restricted to periods of reduced or absent vector activity. Pregnant animals must not be vaccinated, and movement restrictions after vaccination should apply (Alpar *et al.*, 2009). The OIE Terrestrial Animal Health Code (OIE, 2009a) prescribes that animals must be vaccinated at least 60 days before shipment.

With modified-live virus vaccines the primary aim of vaccination is the protection against disease in areas where BTV is already endemic, not the prevention of virus transmission. Conversely, in the face of a BTV epizootic in a previously unaffected region, the prevention of clinical disease is important, but secondary to the interruption of further spread or establishment of the virus (Alpar *et al.*, 2009).

Live vaccines, however, usually cause viremia in vaccinated animals (Veronesi *et al.*, 2005; Veronesi *et al.*, 2010), if only for a short period of time. This can result in the uptake and spread of vaccine strains by vectors when vaccination is carried out during periods of vector activity. Claims that vaccine strains only cause viremia at levels that are insufficient for vector infection are contentious (Kirkland and Hawkes, 2004). Recently, circulation of two vaccine strains in unvaccinated populations has been reported in Italy and Croatia (Ferrari *et al.*, 2005; Listes *et al.*, 2009).

The commercially available live vaccine strains have been attenuated by passages in heterologous culture systems. It is therefore believed that repeated passages between insects and ruminants can restore the original virulence of the vaccine strain, but so far there is no experimental evidence (Alpar *et al.*, 2009).

Viruses with segmented genomes can exchange genetic information during concurrent infection of the same cell with two or more distinct parental strains. Accordingly, vaccination with replication-competent BTV always carries the risk of reassortment of genome segments with other co-infecting BTV strains. This risk increases even further when vaccine strains are no longer confined to the animals that have actually been vaccinated, but are spread by vectors. There are a number of documented examples of reassortment occurring between bluetongue viruses both within and across serotypes (Oberst *et al.*, 1987; Samal *et al.*, 1987a; Samal *et al.*, 1987b; Stott *et al.*, 1987; de Mattos *et al.*, 1991). A 2002 BTV-16 isolate from Italy was shown to carry segment 5 of the BTV-2 vaccine strain. At this point, the BTV-2 vaccine had never been used in Italy, but BTV-2 and BTV-16 MLV vaccines were in regular use in Israel (Batten *et al.*, 2008b). Since then, the use of MLV vaccines against BTV-2, -4, -9 and -16 in Italy has further contributed to the diversity of the circulating viruses, instead of eradicating BTV from the region (Alpar *et al.*, 2009).

When two distinct parental strains reassort, there are 1,022 (2^{10}-2) possible hybrids when all segments can recombine freely. Segments 2 and 6 cannot be exchanged independently in all cases (Matsuo and Roy, 2009), but cross-neutralizing reassortants have been reported (Cowley and Gorman, 1989). Either way, if one single reassortant comes to dominate an entire outbreak, this suggests a considerable selective advantage (Batten *et al.*, 2008b).

In vitro studies demonstrated that a second virus infecting Vero cells does not contribute genome segments to the progeny of the mixed infection when the

second infection occurs more than a few hours after the first (Ramig *et al.*, 1989). After experimental infection of *Culicoides* midges, however, this interference takes several days to develop. Since fully engorged midges readily feed on another blood meal, their high permissiveness for mixed infections might play an important role in BTV evolution (el Hussein *et al.*, 1989).

Relatedly, multivalent vaccine formulations require attention to possible interference and differences in replication efficiency and immunogenicity between the viruses involved (Jeggo *et al.*, 1986; Alpar *et al.*, 2009). A quadrivalent vaccine (BTV-10, -11, -13 and -17) has been used in deer, mouflon and bighorn sheep in California (McConnell *et al.*, 1985). The South African polyvalent live-attenuated vaccine package[2] (originally developed at the Onderstepoort Veterinary Institute) comes in three bottles, to be injected at three-week intervals. Its bottle A contains serotypes 1, 4, 6, 12 and 14, bottle B serotypes 3, 8, 9, 10 and 11, and bottle C serotypes 2, 5, 7, 13 and 19 (Dungu *et al.*, 2004).

The origin and passage history of the strains in the South African product is diverse, ranging from the original BTV-4 strain isolated by Theiler around 1900, to isolates from Cyprus, Portugal and Pakistan (Alpar *et al.*, 2009). Serotypes 15 to 18 and 20 to 24 are not included in the standard formulation, but vaccine strains of some of these serotypes are available on demand, as are monovalent formulations of strains contained in the polyvalent product.

Individually, the modified-live BTV-8 vaccine strain was shown to protect sheep against challenge with European BTV-8 (Dungu *et al.*, 2008). The use of bottle B of the South African vaccine in Bulgaria in 1999 and 2000 in response to the introduction of BTV-9, however, marks the only official use of the polyvalent vaccine package in Europe (Savini *et al.*, 2008). Interestingly, serological evidence for BTV-8 infection of sentinel animals was obtained from Bulgaria in 2006, roughly coinciding with its introduction to northwestern Europe. No virus or RNA was found, therefore the relevance of these reports cannot be determined (Oura *et al.*, 2006). The isolates of BTV-8 from elsewhere in Europe, however, do not match the modified-live vaccine strain (Maan *et al.*, 2008).

In other recent campaigns in France, Italy and Spain, only custom monovalent or bivalent live vaccine formulations of South African origin, matched to the serotypes involved in the outbreaks, have been used. No inactivated vaccines were commercially available before 2005 (Savini *et al.*, 2008). Table 3 lists BTV vaccines that have been used in Europe until 2006.

[2] Onderstepoort Biological Products, Onderstepoort, South Africa; http://www.obpvaccines.co.za/prods/41.htm

Country	Monovalent modified-live virus vaccines			
	BTV-2	BTV-4	BTV-9	BTV-16*
France (Corsica)	2000-2002			2004
Italy	2002-2006	2004-2006	2004-2006	2004
Portugal		2005-2006		
Spain	2000-2001	2004-2006		
Country	Bi- or polyvalent modified-live virus vaccines			
	BTV-2/4	BTV-2/9	Bottle B**	
Bulgaria			1999-2000	
France (Corsica)	2003-2004			
Italy	2004-2006	2002-2006		
Spain	2003			
Country	Inactivated vaccines			
	BTV-2	BTV-4	BTV-2/4	
France (Corsica)	2005		2006	
Italy			2005-2006	
Portugal		2005-2006		
Spain		2005-2006		

Table 3: Bluetongue vaccines used in Europe until 2006 (Savini *et al.*, 2008). *Use of the BTV-16 MLV vaccine was discontinued over concerns about its attenuation. **Contains BTV-3, -8, -9, -10 and -11.

A monovalent modified-live virus vaccine against serotype 16 had been included in a 2004 campaign in Italy, but its use has been discontinued due to insufficient attenuation of the strain (Monaco *et al.*, 2006). In late 2004, BTV-16 with 100% segment 5 homology to the vaccine strain was isolated in Croatia, where the vaccine had never been used. There was no record of relevant livestock movements, and based on meteorological data, a wind-borne dispersal of midges infected with the vaccine strain was suggested (Listes *et al.*, 2009). Interestingly, isolates from the BTV-16 outbreak that had prompted the use of the vaccine in the first place were found to be closely related to the vaccine strain as well. This suggests a recent common origin, possibly in a BTV-16 vaccination campaign in Israel a decade earlier (Mertens *et al.*, 2007), where a custom pentavalent vaccine (serotypes 2, 4, 6, 10 and 16) provided by the Onderstepoort laboratory was used (Shimshony, 2004). This chain of events exemplifies the inherent problems of traditional modified-live BTV vaccines. The recently developed reverse genetic systems (Boyce *et al.*, 2008; Attoui *et*

al., 2009), however, might allow the development of safer alternatives based on directed genetic engineering of bluetongue viruses. First strategies have been suggested very recently (Matsuo and Roy, 2009; Roy *et al.*, 2009; Zhang *et al.*, 2010).

Recombinant vaccines, on the other hand, already offer the good immunogenicity of a replicating virus (the non-BTV backbone) without the possibility of reassortment or vector-borne spread, provided the backbone does not productively infect arthropods. Several prototype vaccines have been developed and tested with promising results. The capsid proteins VP2, VP5 and VP7 of BTV-1 were used in vaccination experiments with a recombinant "Western Reserve" strain vaccinia virus, eliciting neutralizing antibody responses and protection against challenge infection (Lobato *et al.*, 1997). Capripox viruses individually expressing VP2, VP7, NS1 and NS3 of BTV-2 completely prevented viremia in goats challenged with the homologous serotype three weeks after combined application, but their protective efficacy in sheep was much lower (Perrin *et al.*, 2007). In another experiment with homologous virus, a canarypox virus vector (based on the commercial ALVAC platform) expressing both outer capsid proteins of BTV-17 fully protected sheep against virulent challenge (Boone *et al.*, 2007). A capripox virus expressing VP7 of BTV-1 even afforded partial protection against a highly virulent *heterologous* BTV-3 challenge in sheep. While all controls died, most vaccinated animals recovered. Viremia was not examined, but all animals displayed signs of bluetongue disease (Wade-Evans *et al.*, 1996). A replication defective canine adenovirus that expressed VP7 of BTV-2, on the other hand, induced a measurable non-neutralizing humoral response, but did not afford clinical protection against homologous or heterologous challenge (Bouet-Cararo *et al.*, 2009).

As far as BTV reassortment and reversion to virulence are concerned, all recombinant pox viruses are considered safe on account of their DNA genome (Alpar *et al.*, 2009). Only the coding regions of BTV segments are transcribed, yielding mRNAs that lack the conserved termini of native BTV RNA. Their role in RNA packaging during bluetongue virion assembly, however, has not been verified (Lymperopoulos *et al.*, 2003). Vaccinia virus is a very common vector for recombinant vaccines. Its wide host range, however, was named as a potential issue for use in the field. Recent studies on recombinant BTV and AHSV vaccines (Calvo-Pinilla *et al.*, 2009b; Chiam *et al.*, 2009), using a modified vaccinia "Ankara" strain rather than the "Western Reserve" strain, however, reported good immunogenicity for a safe backbone that has lost the ability to productively infect mammalian cells (Sutter and Moss, 1992).

In any case, there are numerous possibilities for BTV vaccines that do not involve replicating virus at all. One inherently safe option is vaccination with

incomplete virions or individual virus proteins, an approach generally referred to as “sub-unit” vaccination. Vaccination with the outer capsid protein VP2 alone can protect sheep against challenge with virulent virus of the same serotype (Huismans *et al.*, 1987). The neutralizing antibody titer, however, as well as the level of observed protection are greater when VP2 and VP5, isolated from purified virus particles or heterologously expressed *in vitro*, are used together – a finding that is possibly explained by their close interaction in the virion, the strong conformational dependence of VP2, and the presence of neutralizing epitopes not only in VP2 but also in VP5 (DeMaula *et al.*, 2000; Alpar *et al.*, 2009). Recombinant baculoviruses have long been used to express BTV proteins with good results (French *et al.*, 1989). When expressed on its own, the sub-core protein VP3 can self-assemble, demonstrating that neither the BTV genome nor NS2 is required for virion assembly (Belyaev and Roy, 1993). Adding VP7 further increases strength and rigidity. The helicase protein VP6 is not readily incorporated into these core-like particles (CLP), however, and it is likely that VP6 needs to be associated with BTV RNA in order to be encapsidated (Nason *et al.*, 2004). The absence of the genome apparently reduces the outward pressure on the CLP (Gouet *et al.*, 1999), resulting in a reduced particle diameter. This in turn can interfere with the addition of the outer capsid layer. But in general, virus-like particles (VLP) present structural proteins in the correct conformation and give a good immune response (Roy *et al.*, 1994). There are stability issues, though, and the efficiency of their production is low, resulting in high costs. In the livestock industry, cost is a major factor in vaccine choice. Accordingly, in spite of the technology being available for two decades, there has never been a commercial BTV vaccine based on VLPs (Alpar *et al.*, 2009). These issues are being addressed in ongoing research efforts, however, and new candidate vaccines have been tested successfully (Stewart *et al.*, 2010).

First experiments with inactivated whole virus vaccines, on the other hand, date back as far as 1975 for BTV (Parker *et al.*, 1975), and even longer for AHSV (Mirchamsy and Taslimi, 1968). Inactivated vaccines offer significant advantages over attenuated vaccines because the absence of replicating virus eliminates concerns about viremia, vector transmission and reversion to virulence and there is no danger of fetal infection or reassortment. Procedures for inactivating bluetongue virus using formalin, β-propiolactone, binary ethylenimine or gamma radiation (Campbell *et al.*, 1985) and administration with different adjuvants have been extensively reported (reviewed by SCAHAW, 2000; Ramakrishnan *et al.*, 2006), but rigorous quality control must be applied to ensure that every vaccine batch is adequately inactivated (Stewart *et al.*, 2010).

Experimental inactivated vaccines against serotypes 3, 4 (Parker *et al.*, 1975), 11 and 17 (Berry *et al.*, 1982) had been shown to be immunogenic, but their protective efficacy was not evaluated. Good efficacy results have been reported for monovalent inactivated vaccines directed against serotypes 2, 4, 11 and 16 and a bivalent vaccine directed against serotypes 2 and 4 (Stevens *et al.*, 1985; reviewed by Savini *et al.*, 2008; Hamers *et al.*, 2009b; Savini *et al.*, 2009). More than 25 years after the study by Parker *et al.* (1975), the first commercial inactivated vaccine became available (Alpar *et al.*, 2009). This BTV-2 vaccine was also the first inactivated vaccine to be used in the field after the emergence of BTV in Europe (Savini *et al.*, 2008).

In 2008, on account of the rapid spread and high impact of BTV-8 in central Europe, the EU and Switzerland agreed on a large-scale vaccination campaign using monovalent inactivated vaccines against that serotype. Four companies offered vaccines based on Dutch and Belgian BTV-8 seed strains, inactivated with β-propiolactone and adjuvanted with aluminum hydroxide and saponin. For the first year, the potential demand across all member states had been estimated at around 250 million doses (Bartram, 2009), and the campaign is still ongoing (Gethmann *et al.*, 2010).

Inactivated vaccines had been selected because of safety concerns with modified-live virus vaccines. Since inactivated vaccines do not contain replicating virus, larger amounts of antigen and suitable adjuvants are required for effective immunization. Together with the perceived need for booster injections, at least in cattle, this leads to increased costs compared to live vaccines (Savini *et al.*, 2008). Still, for the same amount of antigen the production of inactivated whole virus vaccines is considerably cheaper than the production of VLPs. Similar to VLPs, they can be used in strategies to differentiate infected from vaccinated animals (DIVA) (Savini *et al.*, 2008). Depending on the purity of the vaccine formulation they elicit no or only a reduced immune response to non-structural proteins (Alpar *et al.*, 2009). But despite promising experimental results (Barros *et al.*, 2009), no commercial system for BTV DIVA diagnostics is currently available. Most available ELISAs detect antibodies to VP7, which are induced by both BTV infection and vaccination with inactivated whole virus vaccines.

Finally, even where effective vaccines are available and being used, time is needed to build up the immune response. The first immunization with an inactivated vaccine in cattle requires two doses, and the development of stable immunity therefore takes up to eight weeks (Gethmann *et al.*, 2010). This “immunity gap” could be filled by effective antiviral agents (Goris *et al.*, 2008). Up to now, no antivirals against BTV are available, but high-throughput compound screening assays for drug discovery are being deployed (Li *et al.*, 2009).

3 Objectives

When they were first offered in 2008, no independent efficacy data for the inactivated vaccines to be used in the German vaccination campaign had been available. Their short- and long-term efficacy was evaluated in vaccination and challenge experiments in sheep and cattle. In addition, the possible detection of BTV genome after vaccination with an inactivated vaccine was investigated.

No serotype-specific real-time RT-PCR assays for the detection of BTV serotypes with relevance for Germany had been available. Appropriate assays were designed and validated.

The prevalence of BTV-6 infection in northwestern Germany was unknown, and limited information on the virulence of the emerging BTV-6 strain was available. This was investigated in a field survey and animal experiments in cattle, sheep and mice.

The use of blood as an inoculum in BTV animal experiments is often impractical. The suitability of culture-grown virus for challenge experiments was assessed in sheep, and the recently proposed BTV model in interferon receptor-deficient adult mice was tested.

4 Results

The manuscripts are presented in the form accepted for publication.

Each manuscript has its own reference section formatted in the style of the respective journal; references and abbreviations from the manuscripts are not included in the relevant sections at the end of this document. Figures and tables are numbered individually within each manuscript.

The poster “European Bluetongue virus serotypes 6 and 8: Studies on virulence and cross-protection” is reproduced as presented at the 4th European Congress of Virology. A relevant manuscript is in preparation.

Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep

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Abstract

Bluetongue has become a major animal health issue in the European Union. The member states and Switzerland have agreed on a vaccination strategy. Three different inactivated monovalent vaccines against bluetongue virus serotype 8 were selected for the compulsory vaccination program carried out in Germany in 2008. The efficacy of these vaccines was evaluated in a pilot study in sheep immunised under field conditions by clinical, virological and serological examination before and after experimental challenge infection with a BTV-8 field isolate. Antibody levels prior to challenge infection differed between the vaccinated groups, but all seroconverted animals were fully protected against clinical disease and virus replication. Only one vaccinated animal was very weakly positive in the real-time RT-PCR at day 10 after challenge infection, and one seronegative sheep in one of the vaccine groups was not protected.

Introduction

Bluetongue (BT) is a non-contagious, arthropod-borne disease of ruminants and camelids caused by bluetongue virus (BTV) of the genus *Orbivirus* in the family *Reoviridae*. BTV is transmitted by *Culicoides* spp. biting midges. It can cause severe disease, particularly in sheep, and can have considerable economic impact. BT is notifiable to the World Organisation for Animal Health (OIE, Office International des Epizooties). Presently there are twenty-four established serotypes (BTV-1 to -24) with only low levels of cross-protection[1]. A putative new BTV serotype, Toggenburg orbivirus, was recently discovered in Switzerland[2]. Bluetongue was first described in Africa at the beginning of the 20th century, but did not regularly occur in Europe (except Cyprus) before 1998[3]. Since then, at least five serotypes (BTV-1, -2, -4, -9 and -16) have invaded Mediterranean Europe on different occasions. After its introduction in 2006, BTV-8 has caused a severe epizootic in Western and Central Europe, in areas where BTV had never been reported before and which were not considered to be at risk of BTV introduction[4-8]. Smaller incidents involving BTV-6 and -11 that were ascribed to the illegal use of modified live vaccines have been reported in Northern Europe[9]. The route of introduction of BTV-8 is still unclear[10], but it now represents a serious threat to the livestock population of Europe. Vaccination accompanied by ancillary measures such as movement restrictions and surveillance has been demonstrated to be an effective veterinary public health instrument to control the spread of BT[11-13]. The EU member states

and Switzerland agreed on a vaccination strategy to reduce clinical disease and losses in domestic ruminants, to contain the spread of the disease and to facilitate safe trade in live animals[14]. A number of imported modified-live virus (MLV) vaccines against serotypes 2, 4, 9 and 16 had been used in Southern Europe[12] when inactivated vaccines had not been commercially available[15]. Due to the risks associated with MLV vaccines, e.g. insufficient attenuation leading to clinical disease, teratogenicity, spread by vectors with possible reversion to virulence and reassortment of genes with field strains[4,12,16-18], the use of inactivated vaccines is preferred[4,19-22]. Inactivated vaccines directed against serotypes 2, 4 and 16 have already been shown to be generally safe and effective[12,23]. Experimental inactivated vaccines against other serotypes are in development in India and China[13].

In 2008, Germany initiated a compulsory vaccination program against BTV-8 using inactivated monovalent vaccines. Covered by fast-track legislation, vaccinations were scheduled to begin as soon as the newly developed vaccines were available, i.e. before any of the vaccines had obtained EU marketing authorisation[24]. While this procedure is acceptable in the face of serious disease events[25], there is an unequivocal preference for authorised vaccines[26]. When seeking marketing authorisation for veterinary immunologicals with the European Medicines Agency (EMEA), manufacturers need to provide safety and efficacy data. While the EMEA does not require field trials for authorisations of bluetongue vaccines under exceptional circumstances[26], studies to evaluate the safety and efficacy of three inactivated monovalent vaccines (BLUEVAC® 8, CZ Veterinaria S.A., Porriño, Spain; Zulvac® 8, Fort Dodge Animal Health, Naarden, The Netherlands and BTVPUR® AlSap 8, Merial S.A.S., Lyon, France) were commissioned[27] as a prerequisite for the exceptional authorisation in Germany under §17c Tierseuchengesetz (Animal Diseases Act)[28]. After the safety of the vaccines had been assessed in a field study in the German federal state of Mecklenburg-Western Pomerania[28], animals from this field study were used to evaluate vaccine efficacy. The purpose of the present study was to determine the level of protection conveyed by the three vaccines employed in Germany when applied under field conditions against an experimental challenge infection with BTV-8. In addition to a general evaluation of vaccine efficacy, differences in the degree of protection provided by the different vaccines, if any, were to be assessed. For eventual full authorisation, the manufacturers must combine safety and efficacy results from laboratory studies with supportive data from field studies[25].

Materials and methods

Sheep

Twenty-three healthy female German black-headed mutton sheep were obtained from a farm that had participated in the vaccine safety field study in Mecklenburg-Western Pomerania[28]. The farm is situated in a region that had never been affected by BT. All animals had been tested negative for BTV antibodies in the early spring of 2008 before they were included in the vaccine safety study. The animals were randomly selected from the vaccine groups, resulting in a broad age distribution. Seventeen animals were between six and fifteen months of age and six were older than fifteen months. This provides an adequate cross-section of an exposed population in the field. The animals were sequentially numbered and divided into three groups based on the vaccine they had received (table 1).

Group label	Vaccine	Dosage	Second dose	Sheep
controls	saline solution	1 ml	after 3 weeks	1 - 6
C	BLUEVAC® 8	2 ml	no	7 - 12
F	Zulvac® 8 Ovis	2 ml	after 3 weeks	13 - 17
M	BTVPUR® AlSap 8	1 ml	no	18 - 23

Table 1. Vaccines and groups.

Groups C and M consisted of six animals each, group F comprised 5 animals. Six animals from the same farm and breed that had been mock-vaccinated with saline solution were used as controls. These animals were about six months old at the time of challenge. Animals in groups C and M had been vaccinated once three months before challenge infection, sheep in group F had been given the second dose of vaccine that is recommended by the manufacturer three weeks later, i.e. nine weeks before challenge. The animals were subsequently transferred to the BSL-3 facility of the Friedrich-Loeffler-Institut (FLI). They were shown to be free of circulating BTV genome by real-time RT-PCR before challenge infection. During the entire study, rectal body temperatures were measured daily, and the animals were examined for clinical signs by veterinarians.

Vaccines

In the preceding field study, the animals had been vaccinated with one of the three inactivated vaccines intended for the 2008 BT vaccination campaign in Germany[28]. The vaccines in question are inactivated, monovalent BTV-8

preparations in aqueous solution. Seed viruses were European BTV-8 isolates from the 2006 outbreak. Saponins and aluminium hydroxide were used as adjuvants, and vaccines C and F also contain thiomersal. All animals were vaccinated following the instructions provided by the manufacturers (table 1). The vaccines were injected subcutaneously into the lateral thoracic wall. In the field study, vaccinations were carried out by veterinarians under the supervision of the Institute of Epidemiology of the Friedrich-Loeffler-Institute.

Challenge virus

The blood used for challenge infection of the sheep was obtained from a Holstein calf that had been intravenously inoculated with a blood specimen submitted by the veterinary authorities of the district of Aachen, Germany, to the national reference laboratory for BT in August 2007 (submission no. 173/07). The specimen was from a BT outbreak in a sheep herd held close to the Belgian border south-west of Aachen. In accordance with OIE recommendations for BTV challenge experiments[22], the virus was not subjected to any passages in embryonated chicken eggs or cell culture. After inoculation with the specimen, the calf was monitored for viraemia by real-time RT-PCR. A half-litre of blood was taken by jugular venipuncture on day 7 after inoculation in a phase of high-level viraemia. The blood was collected in tubes containing potassium EDTA and stored at 4 °C. Virus was isolated from the blood by tissue culture inoculation and stored for reference.
On the day of challenge infection of the sheep, a 150 ml aliquot of the blood was diluted with an equal volume of sterile phosphate-buffered saline (PBS) and centrifuged. The supernatant was discarded and packed blood cells were washed twice with PBS. Subsequently, blood cells were resuspended in PBS, sonically disrupted and the lysate was divided for injection. The blood preparation was confirmed to be free from bacterial contaminations. The unprocessed blood not used for challenge infection remained in storage for further experiments.

Challenge infection

On day 0 of the study, all sheep were inoculated with 4 ml of the bovine blood preparation at 1.25×10^3 50% tissue culture infective doses ($TCID_{50}$) per ml and 1.22×10^6 BTV genome copies per ml. Every animal received two subcutaneous injections of 2 ml in the bald axillar regions of both sides. The infective dosage was determined by end-point titration on baby hamster kidney (BHK)-21 C13 cells (RIE179, Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems, Germany). BTV genome content was measured by real-time RT-PCR.

Blood samples

Whole blood samples from all animals were taken on days 0, 3, 4, 5, 6, 7, 10, 12, 14, 17 and 21, and serum samples on days 0, 7, 14 and 21 post challenge infection (dpi). Blood was drawn by jugular puncture and collected in tubes containing potassium EDTA or clot activator, respectively (Monovette, Sarstedt, Nümbrecht, Germany). Samples were stored at 4 °C until analysis.

RNA detection and quantification

Total RNA from whole blood samples was extracted using a commercial kit (NucleoSpin 96 RNA, Macherey-Nagel, Düren, Germany) in an extraction robot (Freedom EVO, Tecan, Männedorf, Switzerland). The amount of bluetongue viral genome in samples and the inoculum was determined using two real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays, specific for BTV or BTV-8, respectively. The BTV-8-specific assay (Hoffmann et al., unpublished) amplifies a fragment of the gene encoding the outer shell protein VP2, which is highly variable between serotypes. The BTV-specific assay amplifies a highly conserved sequence at the 5' end of BTV genome segment 5[29]. One-step RT-PCR was performed for 10 minutes at 50 °C and 5 min at 95 °C followed by 42 cycles of 15 seconds at 95 °C, 20 sec at 56 °C and 30 sec at 72 °C (iScript One-Step RT-PCR Kit for Probes, Bio-Rad Laboratories, Hercules, CA, USA) in an Mx3005P QPCR system (Stratagene, La Jolla, CA, USA). BTV genome copy number in the inoculum was determined with a calibration curve obtained by concurrent amplification of serial dilutions of a BTV genome standard with known copy numbers. Our implementation of the BTV-specific assay has been favourably evaluated in an inter-laboratory comparison test[30]. Accordingly, we used it to cross-validate the newly developed BTV-8 specific assay. Both assays show highly similar sensitivity.

Serology

Before challenge infection, serum samples were collected from all animals to determine the initial level of BTV-specific antibodies. Changes in antibody levels were monitored by taking further samples at 7, 14 and 21 dpi. Undiluted sera and serial two-fold dilutions up to 1:512 in PBS were tested in the ELISA. Antibody levels against the BTV major core protein VP7 were determined with two commercially available ELISA kits. A competitive assay (ID Screen Bluetongue Competition, ID VET, Montpellier, France) and a double-antigen sandwich assay (Ingezim BTV DR, Inmunología y genética aplicada, S.A., Madrid, Spain) were used according to manufacturers' instructions.

Neutralising titres against BTV-8 in serum samples were determined as described by Savini *et al.*[31], testing serial two-fold dilutions starting with an initial dilution of 1:10. If by this method no neutralising antibodies could be detected in a sample while the undiluted serum had been positive in the competitive ELISA, dilutions of 1:2, 1:4 and 1:8 were also tested for virus neutralisation. Neutralising doses given for samples (ND_{50}) are the reciprocal of the serum dilution that caused virus neutralisation in 50% of the replicates tested, as calculated by the method of Spearman and Kärber[32]. As controls, we used dilutions of sera whose titres had been determined by the OIE reference laboratory for BT at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Teramo, Italy.

Results

Serology

Before challenge infection, all but one vaccinated sheep had BTV-specific antibodies; one sheep in group C (no. 12) and all sheep in the control group were negative in all serological assays. Serial dilutions of sera taken immediately before challenge infection showed the highest antibody titres for group F (two-shot vaccination). In the competitive ELISA, sera from all sheep were negative at a dilution of 1:16. The more sensitive sandwich ELISA scored positive in dilutions of up to 1:256 (figure 1a).

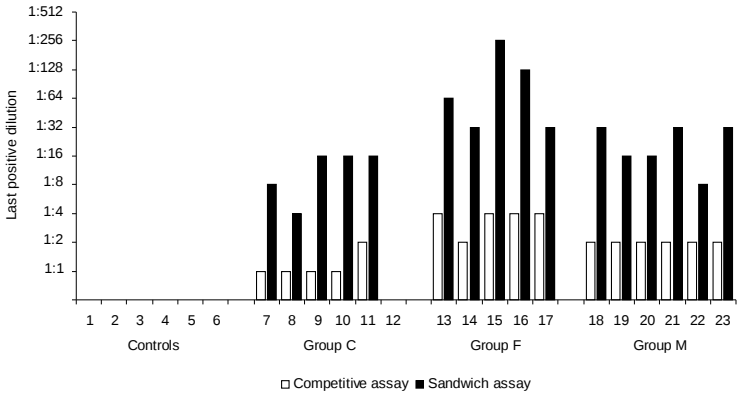

Figure 1a. VP7 antibody titres of pre-challenge sera. Serial two-fold dilutions were evaluated with two different commercial ELISA kits.

On average, VP7 antibody levels (i.e. optical densities measured in the sandwich ELISA) in undiluted pre-challenge sera were lowest for the sheep in group C (figure 1b), even when the negative result for sheep 12 was not taken into account.

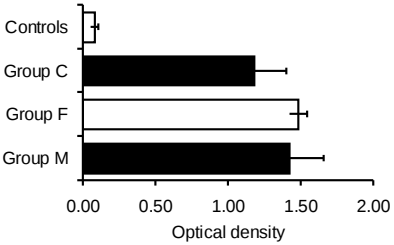

Figure 1b. Average antibody levels of animals grouped by vaccine; in undiluted pre-challenge sera as measured by the sandwich ELISA. The group C average does not include sheep 12.

On day 7 after challenge infection, all vaccinated animals except sheep 12 showed a marked boost in antibody levels in the competitive ELISA, and all controls and sheep 12 had seroconverted by day 14 at the latest (data not shown).

The ELISA results were corroborated by the BTV-8 neutralisation assay. Before challenge, the controls and sheep 12 had no neutralising antibodies, neither did one sheep in group M (sheep 21) that had been positive in the competitive ELISA. On average, pre-challenge neutralising titres were highest in group F, neutralising antibody levels of all vaccinated animals except sheep 12 increased markedly in the first week after challenge, and all sheep displayed neutralising antibodies not later than two weeks after challenge (table 2).

Sheep	Vaccine	Neutralising dose 50%			
no.	group	0 dpi	7 dpi	14 dpi	21 dpi
1	controls	<10	<10	40	80
2	controls	<10	2	240	320
3	controls	<10	2	120	120
4	controls	<10	<10	60	120
5	controls	<10	<10	10	10
6	controls	<10	<10	60	60
7	C	4	320	480	480
8	C	3	160	640	960
9	C	2	160	480	480
10	C	2	240	480	640
11	C	8	320	480	640
12	C	<2	<2	120	160
13	F	15	160	1280	640
14	F	2	120	640	480
15	F	80	120	1280	960
16	F	40	320	320	480
17	F	15	80	320	240
18	M	2	160	120	160
19	M	4	120	240	160
20	M	15	320	320	960
21	M	<2	640	240	240
22	M	6	160	320	480
23	M	15	480	1280	960

Table 2. Results of the BTV-8 neutralisation assay. Neutralising doses given for samples (ND50) are the reciprocal of the serum dilution that caused virus neutralisation in 50% of the replicates tested.

RNA detection

In five of six sheep in the control group (all but sheep 5), viral genome copies were first detected on day 3 using real-time RT-PCR assays. Genome copy numbers peaked on day 10 and viral genome remained present until the end of the sampling period (figure 2).

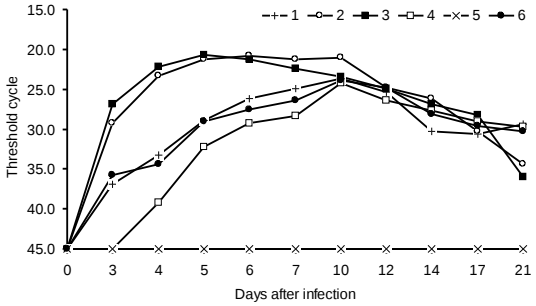

Figure 2. Threshold cycle (C_t) values of a BTV-8-specific real-time RT-PCR, results for the sheep of the control group. The BTV-specific assay gave similar results (data not shown). Negative results are shown as a C_t of 45.

Sheep 12 (group C) demonstrated viral replication at similar levels to the non-vaccinated animals in both the BTV-8 specific and the BTV-specific assays. On day 10, sheep 9 in group C was tested positive in the BTV-8 specific real-time RT-PCR, albeit at a threshold cycle of 36 which is close to the detection limit. No BTV genome was detected in any other vaccinated animal (table 3).

Sheep no.	Vaccine group	Days after infection										
		0	3	4	5	6	7	10	12	14	17	21
7	C	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
8	C	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
9	C	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	36.27	No Ct	No Ct	No Ct	No Ct
10	C	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
11	C	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
12	C	No Ct	32.17	27.58	24.35	21.49	22.47	23.92	27.50	29.39	27.24	29.46
13	F	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
14	F	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
15	F	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
16	F	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
17	F	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
18	M	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
19	M	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
20	M	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
21	M	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
22	M	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
23	M	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct

Table 3. Threshold cycle (C_t) values of a BTV-8 specific real-time RT-PCR, results for vaccinated sheep.

Clinical observations

Sheep in the control group showed mild clinical symptoms of disease, including an elevated body temperature between days 5 and 12 after challenge infection as compared to vaccinated animals (figure 3). One animal in the control group (no. 2) had a marked facial oedema and increased nasal discharge on days 7 to 9; another animal (no. 4) displayed forced breathing in the same time period. No symptoms of bluetongue disease[33] were evident in any of the vaccinated animals.

Post-mortem examinations

At post-mortem examination at the end of the study (25 days post infection), sheep 2 of the control group showed a focal, 5 mm in diameter, intramural

haemorrhage of the pulmonary artery, 1.5 cm above the origin. The medulla of the right superficial cervical lymph node showed multifocal to coalescent hyperaemia and haemorrhage in sheep 4 of the control group. Several sheep of different groups had intimal melanosis of the pulmonal artery which had to be differentiated from haemorrhage (data not shown). There were no lesions consistent with bluetongue disease[33] in any vaccinated animal.

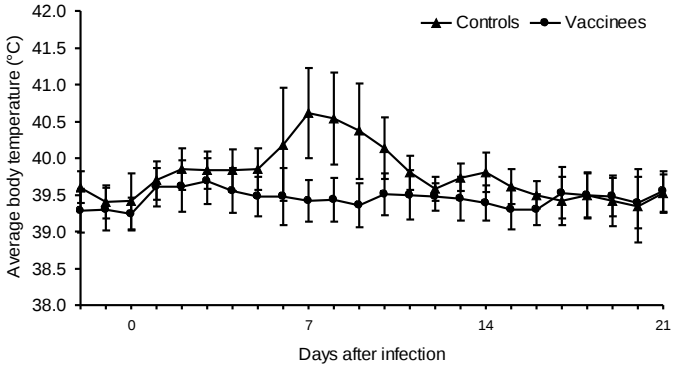

Figure 3. Rectal temperatures of controls and vaccinated animals (group averages with standard deviation) after challenge infection with BTV-8.

Discussion

Good efficacy results for bluetongue vaccination in sheep have been reported for monovalent inactivated vaccines directed against serotypes 2, 4, 11 and 16 and a bivalent vaccine directed against serotypes 2 and 4[12,34]. In these studies, two-shot vaccination evoked neutralising antibodies in most cases and consistently prevented both viraemia and clinical disease. Two doses of an experimental inactivated vaccine directed against serotype 18, on the other hand, reportedly failed to protect sheep from viraemia after challenge[35]. There is little precedent for challenge experiments with sheep that received only a single dose of an inactivated vaccine. Experimental inactivated vaccines against serotypes 3, 4, 11 and 17 have been successfully tested for their immunogenicity when applied once, but no challenge infections were carried out[36,37]. An inactivated vaccine against BTV-2 led to 100% seroconversion in sheep following the first injection, and full protection against challenge infection has been reported[38]. Nevertheless, up to now there are no published data for inactivated vaccines against BTV-8.

Within the scope of the present study, all three vaccines protected sheep against challenge infection with a German BTV-8 isolate, two of the vaccines even after a single-shot application as recommended by the manufacturers. Immunity against BT provided by inactivated vaccines is suspected to be only transient, especially after a single injection[1]. Cattle usually require a booster vaccination for durable immunity[12]. For inactivated vaccines against other serotypes, protection was shown to last at least six months for sheep inoculated once, and up to twelve months after two doses[12]. Since a decline of vaccine-induced antibody levels can be expected over time, and at the same time no further vaccinations took place in Germany in 2008, it is unclear whether sufficient immunity to BTV infection will remain in single-vaccinated sheep herds over a period of 12 months until revaccination in 2009. This is being addressed in an ongoing study.

Presently, serological analyses of samples collected before challenge infection showed a positive effect of two-shot vaccination on antibody levels. Antibody levels measured in the ELISA do not necessarily correspond to levels of neutralising antibodies. The latter are serotype-specific antibodies directed against structural proteins of the outer capsid (mostly VP2), while according to information provided by the manufacturers both ELISA kits used here detect BTV group-specific antibodies against an inner capsid protein (VP7). This is the case for most commercially available BT ELISA kits[22]. The precise relationship between VP7 and VP2 antibody levels induced by inactivated vaccines still needs to be determined. In sheep that had been vaccinated twice (as recommended by the manufacturer of vaccine F), antibodies were detectable by

the sandwich ELISA at dilutions of up to 1:256, while sheep that had been vaccinated only once turned negative in the 1:64 dilution at the latest. Results obtained with the less sensitive competitive ELISA show a similar trend, and the neutralisation assay also detected the highest titres in group F. However, animals of the two other vaccinated groups that initially had lower antibody titres were also protected against challenge infection, including sheep 21 (group M) that did not have any detectable neutralising antibodies at all. Protection against challenge infection in the absence of neutralising antibodies has previously been reported for other inactivated vaccines[34,39]. In the present study, antibody levels before infection as well as the dynamics of seroconversion after challenge infection differed between diagnostic assays. Overall, the sandwich ELISA exhibited the greatest sensitivity and should therefore be used to verify the vaccination status of animals.

Still, one sheep in group C (no. 12) turned out completely seronegative for BTV at day 0 in all serological assays. The existence of non-responders, i.e. immunocompetent individuals that fail to mount an antibody response after vaccination with certain vaccines, is well established in humans[40]. For ruminants, individual differences in immune responsiveness have been shown[41]. Still, it seems more likely that sheep 12 had not received a correct dose of the vaccine, particularly because sheep in that group were only vaccinated once, increasing the possible influence of human error. The animal was not protected against challenge infection as shown by the high-level viraemia after challenge infection. There was no booster effect on antibody levels after challenge. Similar to sheep in the control group, sheep 12 seroconverted two weeks after challenge. Conversely, sheep 21 that was positive in the ELISA but initially did not have detectable neutralising antibodies displayed a marked increase of antibody levels as early as 7 days after challenge infection.

In one vaccinated animal, sheep 9 in group C, BTV genome was detected with a threshold cycle of 36 in the highly sensitive BTV-8 specific real-time RT-PCR on day 10, but the animal was negative in subsequent samplings. The sheep had BTV-specific antibodies prior to infection, and did not show any clinical signs of BT at any time. Monitoring the level of viraemia in vaccinated animals after challenge infection is considered the most effective way to evaluate vaccine efficacy[12,26]. However, in the light of the low amount of viral genome detected on only one day and the absence of clinical disease, the potential epidemiological relevance of this finding is questionable. At this low level of circulating viral genome, the presence of infectious virus can usually not be demonstrated. An insect vector would be highly unlikely to become infected[19,42,43].

Unexpectedly, one unvaccinated sheep in the control group (no. 5) did not display detectable virus amplification after challenge infection, but seroconverted by day 14 as measured in the competitive ELISA. Interlaboratory proficiency tests have proved that the real-time RT-PCR is adequately sensitive[30]. The animal first displayed neutralising antibodies on day 14, but at a lower level than the other sheep in its group. The emergence of BTV-specific antibodies substantiates that the animal had in fact been inoculated with challenge virus. It appears unlikely that the failed infection was caused by an insufficient dose of infectious virus, as shown by the reisolation of virus from the inoculum. BTV isolation in cell culture is less sensitive than inoculation of sheep[29,44], but the injected dose was found to be over 1000-fold higher than the minimum culture infectious dose determined by end-point titration. Breed-related differences in susceptibility to BTV, including the development of viraemia, have been documented[33,35], so the absence of detectable viraemia in this animal as well as the generally mild symptoms displayed by viraemic animals in the control group could be due to individual differences in innate immune response together with a generally reduced susceptibility of the used breed. This probably could have been avoided by choosing a more susceptible breed such as Poll Dorset[45]. However, this was pre-empted by the design of the precursive safety study, which required a large number of animals. Thus, the availability of the breed serves as an indicator of its importance to the German livestock industry, justifying its selection for the study.

In conclusion, all inactivated vaccines against BTV-8 included in this study showed good efficacy in a challenge experiment. Their safety has already been demonstrated[28,46]. Both findings are in line with results reported for inactivated vaccines directed against other serotypes, namely 2, 4, 11 and 16. Unique in the present study, however, is the use of animals vaccinated against BTV-8 in the field, as opposed to conducting both vaccination and challenge experimentally. Mass vaccinations in the field are logistically challenging, and deficiencies in their implementation can impair both individual and herd immunity, while experimental vaccinations provide unrealistically ideal conditions. Thus, our study presents a more realistic assessment of the efficacy of the selected BTV-8 vaccines under field conditions.

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Evaluation of humoral response and protective efficacy of three inactivated vaccines against bluetongue virus serotype 8 one year after vaccination of sheep and cattle

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Abstract

The long-term efficacy of three commercially available inactivated vaccines against bluetongue virus serotype 8 (BTV-8) (BLUEVAC® 8, Zulvac® 8, and BTVPUR® AlSap 8) was evaluated in a seroprevalence study and challenge experiments. Seroprevalences one year after vaccination ranged from 75% to 100%. In two infection experiments, groups of vaccinated sheep and cattle selected either randomly or for low antibody levels were challenged with a European BTV-8 strain twelve months after vaccination. With two exceptions, all animals, including those with low antibody levels prior to challenge, were protected from viral replication and clinical disease even at low initial antibody levels. Vaccination of susceptible ruminants in yearly intervals is thus considered an adequate scheme for BTV-8 control in Europe.

Introduction

Bluetongue disease (BT) is a notifiable animal disease transmitted to ruminants and camelids by *Culicoides* spp. midges. The disease is caused by a virus of the genus *Orbivirus*, family *Reoviridae*. *Bluetongue virus* (BTV) had previously been considered exotic to central Europe; however, first cases in commercial livestock caused by viruses of serotype 8 were reported in the Netherlands, Belgium and Germany in 2006. The successive spread of the virus was of epizootic character[1-2].

On account of the high economic losses caused by disease outbreaks and by transport restrictions imposed thereafter, a large-scale vaccination scheme was introduced to European Union member countries and Switzerland[3]. Modified-live vaccines (MLV) have been used extensively in areas such as South Africa and Italy[4-5] where multiple BTV serotypes circulate. However, a live attenuated BTV-2 vaccine strain has been spread to naïve populations by infectious vectors[6]. Furthermore, vaccination with a live BTV-16 vaccine in Italy was discontinued due to unpredictable clinical effects attributed to insufficient attenuation of the strain[7]. Therefore, for safety reasons, it was decided to employ only inactivated monovalent vaccines for the mass vaccination schemes against serotype 8.

In order to evaluate the safety of the used vaccines in sheep and cattle, a large-scale field study on three of the inactivated commercial vaccines was conducted by the State Office for Agriculture, Food Safety and Fisheries (LALLF) of Mecklenburg-Vorpommern, Germany and the Friedrich-Loeffler-Institut (FLI) [8], showing only minor adverse effects of vaccination. Furthermore, all three vaccines induced good seroconversion rates as well as good protective

immunity, as shown by a challenge experiment in sheep three months after vaccination[9].
The aim of the present study was to evaluate the protective immunity in sheep and cattle one year after vaccination. In addition, protective immunity in animals with a low remaining antibody response to vaccination was examined.

Materials and methods

Animals

Animals used for the study were Holstein-Friesian dairy cattle and German Black-headed Mutton sheep. All animals included in the challenge experiments originated from farms that had participated in the BTV-8 vaccine field study in Mecklenburg-Vorpommern in 2008[8].
The controls were between six and ten months old at the time of challenge, and vaccinated animals were between seventeen months and four years of age. Vaccination had been conducted according to the manufacturers' recommendations with three commercially available inactivated monovalent vaccines one year previously. The vaccine preparations used (Table 1) are labelled A, B and C in the text. Cattle had been vaccinated twice in three-week intervals, sheep had been vaccinated either with a single dose (vaccines A and B) or revaccinated after three weeks (vaccine C).

Group	Vaccine	Manufacturer
A	BLUEVAC® 8	CZ Veterinaria S.A. Porriño, Spain
B	BTVPUR® AlSap 8	Merial S.A.S. Lyon, France
C	Zulvac® 8 Bovis Zulvac® 8 Ovis	Fort Dodge Animal Health Naarden, The Netherlands

Table 1
Vaccines used in the study.

The participating farms were situated in a region in the east of Germany where no significant BTV activity has been recorded. After the 2008 vector season, all animals on the farms, including unvaccinated young stock, were screened by real-time RT-PCR to verify that no vector-borne introduction of BTV had occurred. Due to the passive association of BTV with erythrocytes, viral

genome is detectable for several months after infection[10]. A single screening of the whole farm was therefore considered appropriate.
One year after vaccination, 623 cattle and 223 sheep from the 2008 vaccine safety study remained on the three participating farms. In the safety field study, the animals had been separated into two age groups, juveniles (aged three to twelve months at the time of vaccination) and adults (older than twelve months). Animals in these groups now were between fifteen months and two years or over two years old, respectively. The animals were evaluated for BTV-specific antibodies (cELISA, Bluetongue Antibody Test Kit, VMRD, Pullman, WA, USA) in an ISO 17025-accredited laboratory (LALLF, Rostock, Germany) using the 50% cut-off value prescribed by the manufacturer. From these data, the seroprevalence one year after vaccination (the percentage of vaccinated animals positive in the competitive ELISA) was calculated.
The variance of antibody levels within groups twelve months after vaccination was evaluated for 57 bovines and 55 ovines in vaccine group A, 55 bovines and 98 ovines in vaccine group B and 57 bovines and 70 ovines in vaccine group C. Variances were compared by one-sided F tests. P-values of less than 0.01 were considered significant.
At the conclusion of this study, all animals remaining on the farms were revaccinated and are not available for further experiments.

Challenge virus

The virus used for challenge was obtained from the reference virus collection of the German National Reference Laboratory for Bluetongue and was consistent with experiments conducted previously[9]. Briefly, a BTV-8 strain isolated in Germany in 2007 was passaged once in cattle, whole blood was obtained and blood cells were washed three times with phosphate buffered saline. Blood cells were stored at 4 °C until further use and ultrasonically disrupted on the day of challenge. Characterization of the challenge blood included determination of tissue culture infectious doses 50% ($TCID_{50}$) by assessment of cytopathic effect 5 days after infection of Vero cells and calculation of the number of genome copies by quantitative RT-PCR using a dilution series of in vitro transcribed RNA as a standard. Additionally, the challenge inoculum was screened for bacterial contaminants.
A volume of 4 ml of infectious blood containing 2.0×10^5 $TCID_{50}$ and 1.2×10^7 BTV genome copies was used per animal in the first experiment. Challenge virus was subcutaneously injected in four portions (two on each side) in the shoulder region of cattle and the hairless axillary and inguinal regions of sheep in order to target multiple lymph nodes. Virus was retitrated after infection to guarantee consistent titres.

Challenge experiments

Animals were screened prior to infection for serum antibodies by a competitive enzyme-linked immunosorbent assay (cELISA, VMRD) and a serum neutralization test (SNT)[11]. They were confirmed to be free of circulating BTV genome by real-time RT-PCR and were then challenged with a German BTV-8 isolate as specified above. A clinical evaluation was conducted daily and rectal temperatures were measured for 28 days post infection. With the exception of the euthanized cow (see below), the animals did not undergo a pathological examination at the end of the experiment.
EDTA-treated blood samples for real-time RT-PCR and virus isolation were taken immediately before challenge and at days 3, 5, 7, 10, 12, 14, 18, 21, 25 and 28 after infection. Serum samples for evaluation of BTV-specific antibodies by competitive ELISA were taken in weekly intervals. The level of neutralizing antibodies to BTV-8 was determined by SNT for samples taken immediately before infection and 28 days later.

Experiment #1

In the first experiment, randomized groups of six bovines and ovines from each vaccine group (A, B and C) as well as six unvaccinated control animals of both species were selected. Since the challenge experiments were conducted in the BSL-3 facility of the FLI on Insel Riems, all animals had to be transported from their farms of origin. Control sheep came down with increased body temperatures after transport and were treated with antibiotics (25 mg/kg Enrofloxacin, Baytril®, Bayer HealthCare, Leverkusen, Germany) over a four-day period.

Experiment #2

In a successive experiment, groups of five bovines and three to five ovines from each vaccine group were chosen for challenge based on their cELISA results. Animals with low levels of antibodies prior to challenge were selected. Apart from that, the experimental design was kept in concordance with experiment #1. One bovine was treated with antibiotics due to transport-related injuries (Florfenicol, Nuflor®, Intervet, Unterschleißheim, Germany; Enrofloxacin, Baytril®) but did not recover and was euthanized on day thirteen of the experiment.

Real-time RT-PCR and virus isolation

All EDTA blood samples were evaluated by real-time quantitative RT-PCR targeting a BTV-8 specific sequence on the VP2 gene[12]. As internal control, a β-actin specific sequence was amplified in the same reaction tube[13]. Furthermore, to screen for other BTV serotypes, samples taken on day 10 post infection were evaluated by a "pan-BTV" real-time quantitative RT-PCR targeting a conserved segment 5 (S5) sequence[13].
All RT-PCR protocols were conducted on Agilent-Stratagene (La Jolla, CA, USA) real-time cyclers (Mx3000P® and Mx3005P®) using a commercial RT-PCR kit (iScript® One Step RT-PCR Kit, Bio-Rad, Hercules, CA, USA) as previously described[12]. Virus isolation was attempted from RT-PCR positive EDTA-treated blood samples from the beginning (days three to ten post infection) and the end (days 25 to 28 post infection) of the experiments. Isolation was performed on African green monkey kidney cells (Vero, RIE15, Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems, Germany) from 1 ml of whole blood in two passages as described by Clavijo *et al.*[14].

Representation of serological results

In the competitive ELISA, samples are scored by comparing their optical density (OD) to the OD of the negative control. Samples with an OD higher than 50% of the negative control are considered negative. For the sake of the reader, this counterintuitive representation is reversed in this report. OD ratios are subtracted from 100%. Thus, the negative control is assigned a value of 0%, and samples with an OD ratio of over 50% are positive. This is referred to as "%inhibition".

Results

Seroprevalence study

Animals vaccinated with vaccine A displayed a seroprevalence of 91.2% in juvenile and 76.2% in adult sheep one year after a single application, while 100% of the juvenile and 89.8% of the adult sheep vaccinated once with vaccine B were positive in the competitive ELISA. All sheep vaccinated with two doses of vaccine C were seropositive after a one-year period. Bovines, which had all been vaccinated twice, showed seroprevalences well over 90% (Table 2).

Bluetongue virus antibody levels in sheep before and after vaccination as well as twelve months later are summarized in Figure 1. Twelve months after vaccination, the variance of BTV antibody levels in sheep vaccinated with vaccine C (two doses) was significantly lower than in all other groups. At the same time, variance in sheep vaccinated once with vaccine A (one dose) was significantly higher than in all other groups, even when compared to other sheep that received only one dose of vaccine (group B).

Vaccine	Cattle					Sheep				
A		positive	negative	total	seroprevalence		positive	negative	total	seroprevalence
	juvenile	85	2	87	97.70%	juvenile	31	3	34	91.18%
	adult	131	2	133	98.50%	adult	16	5	21	76.19%
	total	216	4	220	98.18%	total	47	8	55	85.45%
B		positive	negative	total	seroprevalence		positive	negative	total	seroprevalence
	juvenile	92	2	94	97.87%	juvenile	39	0	39	100.00%
	adult	122	5	127	96.06%	adult	53	6	59	89.83%
	total	214	7	221	96.83%	total	92	6	98	93.88%
C		positive	negative	total	seroprevalence		positive	negative	total	seroprevalence
	juvenile	90	0	90	100.00%	juvenile	46	0	46	100.00%
	adult	85	7	92	92.39%	adult	24	0	24	100.00%
	total	175	7	182	96.15%	total	70	0	70	100.00%

Table 2
Seroprevalence in vaccinated sheep and cattle one year after vaccination.

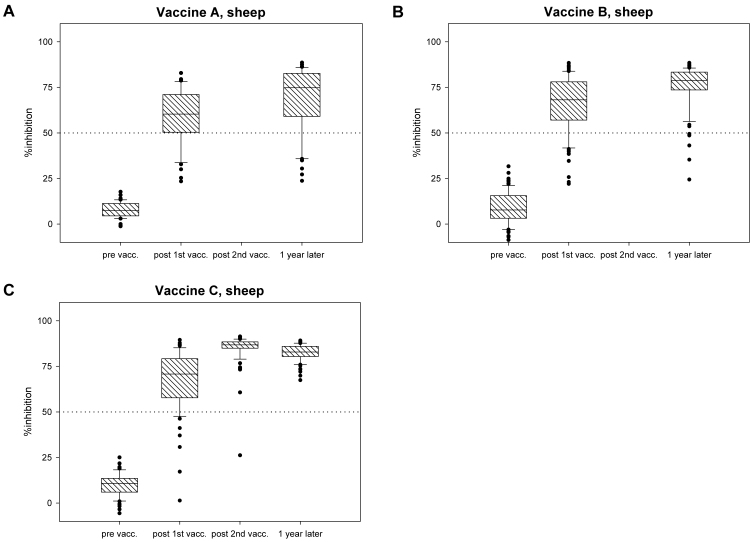


Figure 1A-C
Serological development in sheep over a one-year period as measured by competitive ELISA. The box denotes the median as well as the 25th and 75th percentile. The whiskers denote the 10th and 90th percentile. The circles mark any outliers (individual values outside of that range). The dotted line represents the 50% positive cut-off of the cELISA.

Real time RT-PCR and virus isolation after challenge

Vaccinated animals in experiment #1

In the group of randomly selected vaccinated animals from experiment #1, all of the bovines and all but one of the ovines were protected from viral replication. However, one of the sheep from vaccine group B became RT-PCR positive by day three post infection and continued to show positive results until the end of the experiment (Figure 2).

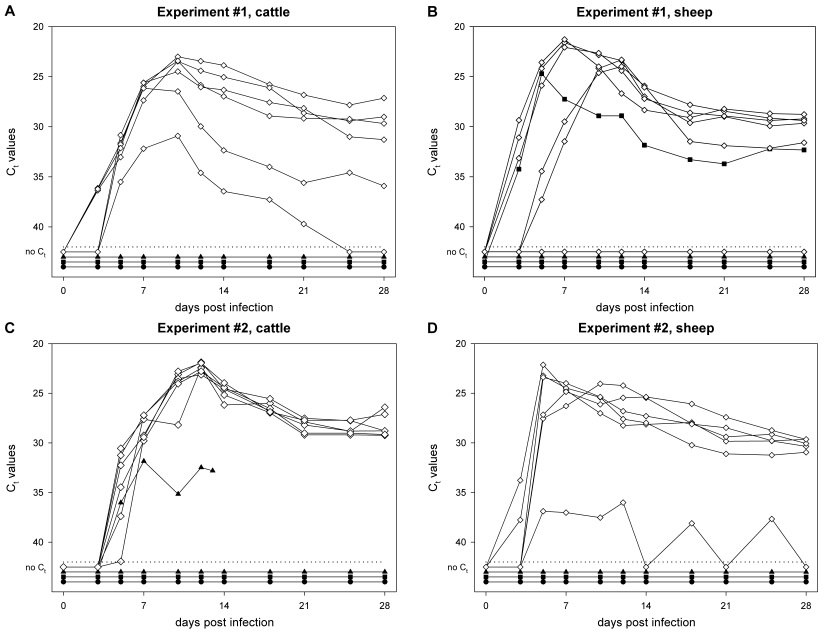


Figure 2A-D
Quantitative RT-PCR results in vaccinated (closed symbols; triangles: group A, boxes: group B, circles: group C) and control animals (open diamonds) after experimental BTV-8 challenge infection.

Vaccinated animals in experiment #2

Results were similar in experiment #2, where selection had been made for individuals with low serum antibody levels. The only vaccinated animal to become positive in the RT-PCR was one bovine from vaccine group A (Figure 2). This animal was in bad general health due to non-BTV related causes (transport injuries; confirmed by a post-mortem examination) and was euthanized on day thirteen of the experiment.

Both the sheep in experiment #1 and the cow in experiment #2 that were RT-PCR positive were the individuals with the lowest serum antibody levels before challenge (17% and 20% inhibition, respectively; see also Figure 3).

Controls

The unvaccinated, seronegative animals from the control groups of both experiments continuously became positive in the RT-PCR between three and five days post infection and remained so until the end of the experiment at 28 days post infection. An exception was made by one of the control sheep in experiment #1, which never turned RT-PCR positive during the entire time period of the experiment. Similarly, one of the control sheep in experiment #2 tested positive only intermittently and displayed much lower quantities of BTV-8 RNA than the other controls (Figure 2); however both these animals seroconverted.

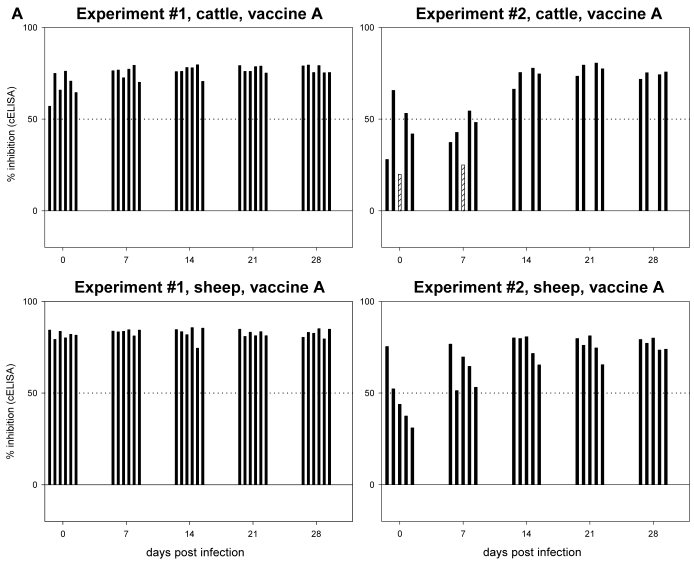
Virus could be isolated from the blood of all RT-PCR positive animals both in the beginning and in the end of the experimental period, but never from the weakly positive control sheep or the euthanized cow (both experiment #2). Retrieval of infectious virus roughly correlated with C_t values. In general, it was not possible to isolate BTV from blood with C_t values above 30 (data not shown).
After challenge, vaccinated animals did not develop clinical signs consistent with bluetongue disease at any time. Mean body temperatures were higher in the control animals for approximately five days during the phase of highest virus load in the blood from seven to ten days post infection. This difference was not as marked in the sheep from experiment #1, where mean body temperatures of the control animals were slightly higher than those of the vaccinated animals from before challenge infection throughout the whole experiment (data not shown).
No BTV serotypes other than BTV-8 were detected in any animal.

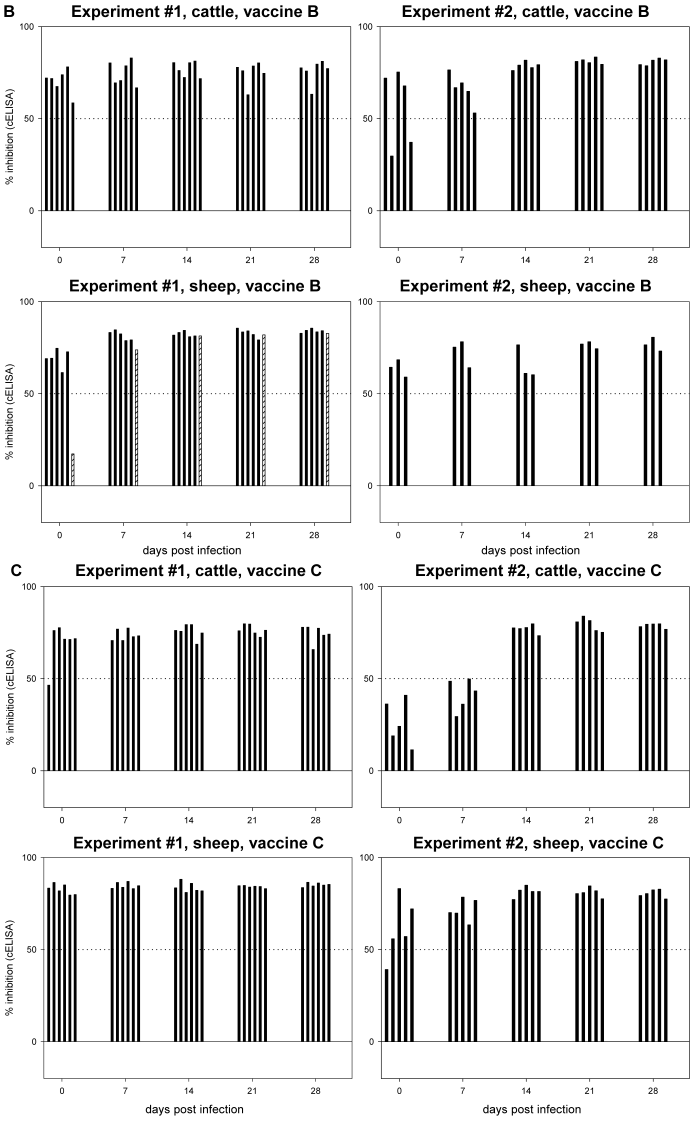
Serology before and after challenge infection

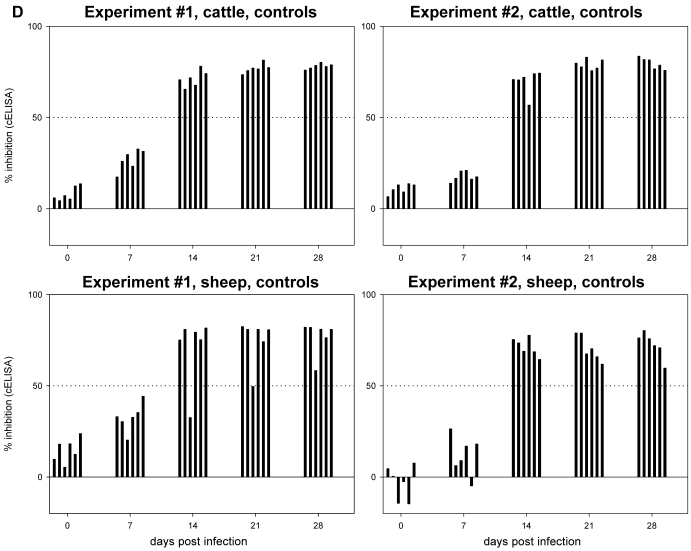
In experiment #1, two of the randomly selected animals (one bovine in group C, one ovine in group B) displayed serum antibody levels below the 50% cut-off value of the competitive ELISA. All other animals were positive.

In experiment #2, animals showed considerably lower initial antibody levels. In ten of fifteen bovines (three in group A, two in group B and five in group C) and four of thirteen ovines (three in group A, one in group C), BTV antibody levels before challenge did not reach the cut-off value of the competitive ELISA. Antibodies detectable by competitive ELISA were present in all vaccinated animals by seven days post infection, albeit still at lower levels than in the serum of animals from experiment #1 at the same time point. By day fourteen post infection, an increase in serum antibodies close to the levels reached by animals from experiment #1 was visible in the ELISA (Figure 3).

Figure 3A-D
Serological results (cELISA) before and after challenge infection (A-C: vaccine groups, D: controls). The dotted line marks the positive cut-off (50%). Serological data of the sheep from experiment #1 and the cow from experiment #2 that were positive in the real-time RT-PCR are shown as striped bars.




B
Experiment #1, cattle, vaccine B
Experiment #2, cattle, vaccine B
Experiment #1, sheep, vaccine B
Experiment #2, sheep, vaccine B
C
Experiment #1, cattle, vaccine C
Experiment #2, cattle, vaccine C
Experiment #1, sheep, vaccine C
Experiment #2, sheep, vaccine C
% inhibition (cELISA)
days post infection
0
7
14
21
28
50
100



In contrast to this, naïve animals of the control groups all showed seroconversion in the competitive ELISA by fourteen days post infection.

A marked boost in neutralising antibody titres was seen at four weeks after challenge (Figure 4). There was no pronounced difference between the vaccine groups; however, SNT titres in the control animals were two- to four-fold lower than those of vaccinated animals at four weeks. The euthanized cow from vaccine group A (experiment #2) had detectable neutralizing antibodies before challenge, while the RT-PCR positive sheep from vaccine group B (experiment #1) did not.

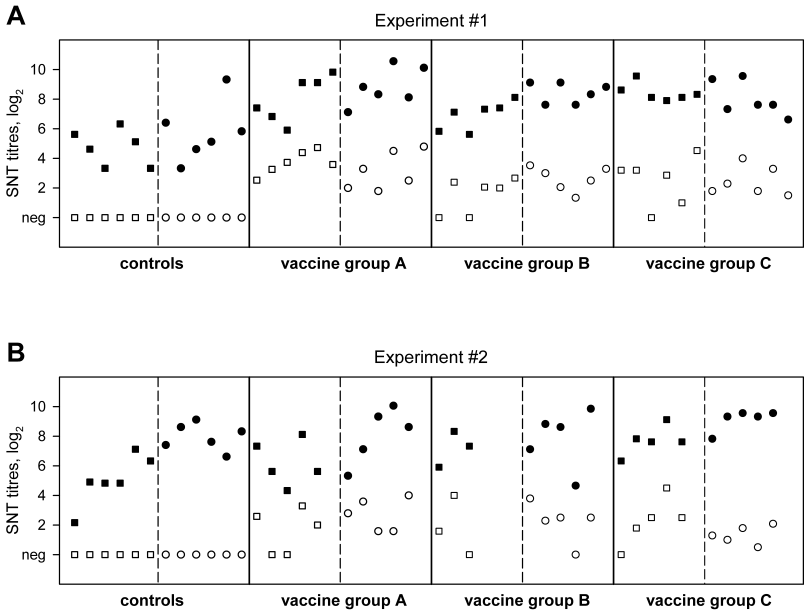


Figure 4A-B
Neutralizing antibodies in vaccinated cattle (circles) and sheep (squares) before (open symbols) and after challenge infection (closed symbols) with a German BTV-8 strain (A: experiment #1, B: experiment #2).

Discussion

The seroprevalence study shows a high degree of seroconversion in vaccinated sheep and cattle one year after vaccination. No marked differences could be observed between the three vaccines in cattle, where all vaccines were applied twice. In contrast, seroprevalence was higher in sheep vaccinated twice with vaccine C as compared to animals vaccinated only once with either one of the other two vaccines. Also, vaccinating sheep twice reduced the heterogeneity of antibody levels within the group. Nonetheless, seroprevalences even after single vaccination with vaccines A or B appear sufficient for protection of sheep. Single-shot efficacy in sheep is important because compliance with double-shot vaccination schedules can be unsatisfactory.

For inactivated vaccines, repeated application is generally considered necessary for the induction of long-term protection, at least in cattle[10,15]. A single application of an inactivated BTV-4 vaccine did not protect cattle from developing viraemia when challenged seven months after vaccination[16]. However, in an experiment in sheep conducted by Hamers *et al.*[17], a single dose of a commercial BTV-2 vaccine has proven to induce protection from challenge for a minimum of 12 months. Recently, a commercial inactivated BTV-8 vaccine that is not included in the present study (Bovilis® BTV8, Intervet International b.v., Boxmeer, The Netherlands) was shown to protect sheep against virulent challenge ten months after vaccination[18].

Given the results of the seroprevalence study, vaccination schemes with yearly intervals between vaccinations are sufficient to maintain stable humoral immune response with the tested batches of inactivated vaccines. This is also supported by the results of the challenge experiments. Nevertheless, batch-to-batch variations could influence efficacy in the field, and consistent quality needs to be confirmed by continuous batch release testing.

In this study, the challenge experiments show a good protective efficacy of all three vaccines in both species tested. Virus replication was virtually eliminated by vaccination in all three vaccine groups. The challenge strain induced no pronounced clinical signs in any of the groups. This is not unexpected for the breed used in the experiment[9]. Still, peak body temperatures after challenge infection were higher in controls than in vaccinated animals. At the time of challenge in experiment #1, the control sheep were recovering from a fever due to transport-related stress and the close contact between animals originating from different holdings. Throughout the experiment, they had a mean body temperature about 0.5 °C higher than the vaccinated sheep and displayed only a slight peak at day seven. The age of the controls might help to explain this observation. Younger sheep generally have a higher body temperature[19], and

often are clinically less affected by BTV-8[20]. Unfortunately, no older BTV-naïve sheep were available for use as controls.

In the challenge experiments, even antibody levels below the cut-off of the competitive ELISA were able to protect most vaccinated animals from virus replication. However, the two cases where virus replication was detectable despite vaccination were both individuals with the lowest serum antibody level in their respective groups. While this does suggest a correlation between serological response and protective efficacy, no definite conclusions can be drawn from observations in only two animals. Furthermore, in the case of the bovine from experiment #2, an additional underlying health problem most likely facilitated the breakthrough of the virus. This suggests that susceptibility to bluetongue virus infection is multifactorial and it is enhanced by underlying general health conditions of non-infectious origin. In the case of the vaccinated sheep that showed low antibody titres and virus replication there are two interpretations: The vaccine was not administered correctly or at an insufficient part of the dose, or the vaccine did not induce a sufficient immune response in this sheep. This could be due to a general non-responsiveness to vaccination or underlying health problems at the time of vaccination which negatively impacted the immune response. Generally speaking, no vaccine can offer 100% of protection in 100% of individuals.

One curious finding that warrants further investigation is the occurrence of less susceptible animals within the same breed, as seen in the control animals that displayed low or no virus replication but eventually seroconverted. Differences in susceptibility based on breed have been described previously[21]. However, our results imply variations within the same breed that co-determine susceptibility to BTV infection. A marked variation in susceptibility within the same breed has also been described by Erasmus[22] without offering a coherent explanation for this phenomenon. It has been postulated that susceptibility to orbiviruses is influenced by intra-species differences in the expression of toll-like receptors[23]. Further research is needed to adequately address this question.

The virus used for challenge is closely related to the vaccine seed strains. Therefore, this study cannot provide information on cross-protection against heterologous BTV-8 strains or other serotypes of BTV. Further research into safe polyvalent vaccines is advisable, since several other serotypes are already present in Europe, and the introduction of new BTV serotypes remains likely[24]. Nevertheless, all three vaccines included in this study protect both cattle and sheep from BTV-8 infection for up to twelve months after vaccination, reliably serving their intended purpose.

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Infectious blood or culture-grown virus: A comparison of bluetongue virus challenge models

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Abstract

The World Organisation for Animal Health (OIE) currently recommends using infectious ruminant blood as challenge inoculum in bluetongue virus (BTV) vaccination and challenge experiments. The use of virus grown in cultured cells is discouraged because culture passages can lead to changes in virus phenotype, including reduced replication efficiency and virulence in the host, while the OIE considers clinical disease in control animals indispensable evidence of successful infection.
In the present study, two groups of five sheep were inoculated with either infectious calf blood lysate or culture-grown bluetongue virus of serotype 8 (BTV-8) (2×10^4 TCID50 and 5×10^5 TCID50, respectively). No pronounced difference in the induction and progression of viraemia as determined by real-time RT-PCR, which is the most objective parameter in the evaluation of vaccine efficacy, was observed. In a second experiment, the virulence of both inocula was confirmed by fatal infection of interferon receptor-deficient mice.
The recent availability of highly sensitive molecular methods for the detection of BTV can finally shift the focus away from clinical disease. For the sake of objective and repeatable BTV challenge experiments, the OIE should reconsider its policy on culture-grown virus.

Introduction

Bluetongue disease (BT) is a non-contagious infectious disease of ruminants and camelids mainly spread by haematophagous *Culicoides* spp. vector insects. There are 24 established serotypes of its causative agent, Bluetongue virus (BTV; *Orbivirus*, *Reoviridae*). After its introduction in 2006, serotype 8 (BTV-8) has caused a major epizootic in Europe. Starting in 2008, mass vaccinations using monovalent inactivated vaccines together with natural immunity after infection led to a dramatic reduction in the spread of BTV-8 (Conraths et al., 2009). The virus however has not been eradicated from Europe and vaccinations will have to continue to effectively control the disease. From a regulatory point of view, the inter-batch variance in potency of licensed vaccines needs to be closely monitored in a sustained vaccination campaign. This calls for repeatable, highly standardised vaccination and challenge experiments.
The present study compares the experimental induction and progression of BTV-8 viraemia in BTV-naïve sheep for two different inocula to assess their suitability for these experiments. Both inocula were also evaluated in the recently published IFNAR-/- mouse model for BTV (Calvo-Pinilla et al., 2009).

Materials and methods

A BTV-naïve Holstein calf was inoculated with a blood sample submitted to the German national reference laboratory for BT in 2008 (Hoffmann et al., 2008). A week later, blood was collected in tubes containing potassium EDTA and stored at 4 °C. On the day of infection of the sheep, an aliquot of the blood was diluted with sterile phosphate-buffered saline solution (PBS) and centrifuged. Packed blood cells were washed twice with PBS and sonically disrupted on ice. The lysate contained 2 x 10^4 TCID50/ml as determined by end-point titration on Vero cells (RIE15, Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institut [FLI], Insel Riems, Germany).
In parallel, BTV-8 isolated directly from the originally submitted sample was propagated in cell culture. Supernatant from the second passage on Vero cells had a virus titre of 5 x 10^5 TCID50/ml. Both inocula were tested free of bacterial contamination. After inoculation of BTV-8 vaccinated sheep, no BTV replication was detected with a "pan-BTV" assay (Toussaint et al., 2007), confirming the absence of other BTV serotypes in the inocula (data not shown).
All animal experiments were conducted in the BSL-3 facility of the FLI on Insel Riems. Ten BTV-naïve yearling German Black-headed Mutton sheep were assigned to two groups of five animals each. Sheep in group A were subcutaneously injected with 4 ml of freshly prepared lysate of infectious calf blood, while sheep in group B similarly received 1 ml of cell culture supernatant.
The animals were monitored for clinical signs every day, rectal body temperatures were measured daily and whole blood and serum samples were taken at days 3, 5, 7, 10, 12, 14, 18, 21, 25 and 28 after infection. Differences in mean body temperature between the groups were examined with Welch's t-test using the R statistical package (version 2.9, http://www.r-project.org). A p-value of less than 0.05 for the null hypothesis was considered significant.
Viral RNA was extracted from whole sheep blood samples using a commercial kit (NucleoSpin 96 Virus, Macherey-Nagel, Düren, Germany) in an automated liquid handling workstation (MICROLAB® STAR, Hamilton, Bonaduz, Switzerland). BTV-8 genome load in the samples was determined by serotype-specific real-time quantitative reverse transcription PCR (RT-qPCR) as described previously (Hoffmann et al., 2009). Again, virus isolation was performed on Vero cells (Clavijo et al., 2000). At weekly intervals, serological data was collected using a commercially available double recognition (sandwich) ELISA kit (PrioCHECK® BTV DR, Prionics Deutschland GmbH, Planegg-Martinsried, Germany) and serum neutralisation assay using homologous virus.

In a second experiment, calf blood lysate and cell culture supernatant were intraperitoneally injected into adult interferon receptor-deficient IFNAR-/- mice (Calvo-Pinilla et al., 2009) and C57BL/6 wild type mice (500 µl per animal). Groups of four IFNAR-/- mice were given either one of the undiluted preparations or dilutions of 1:100 in sterile PBS. Only three IFNAR-/- mice received the diluted culture supernatant, and C57BL/6 mice were only given the undiluted preparations. IFNAR-/- control groups were injected with BTV-negative cattle blood or cell culture supernatant.

Results

In the first sample taken three days after challenge, two out of six sheep in the group inoculated with infectious blood (group A) and all sheep in the culture-grown virus group (B) scored positive in the RT-qPCR. All sheep had detectable viral RNA in the next sample taken on day five. Viral genome remained detectable in all animals until the end of the study at day 28 (see figure 1).

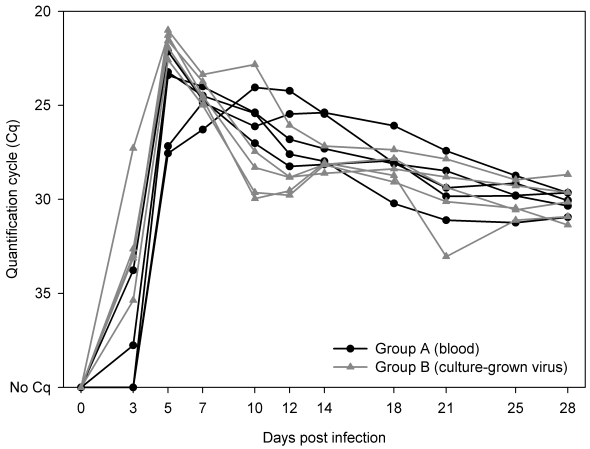

Figure 1: RT-qPCR data for sheep infected with BTV-8.

Virus was reisolated from samples from all sheep in group B starting on day three. In group A, virus isolation was successful in one sample from day three, and in all samples from day five post infection. All sheep remained viraemic for at least 18 days after challenge (data not shown).

The average rectal body temperature in group B peaked at day seven at 0.9 °C over the pre-challenge mean (standard deviation [S.D.] 0.8). The group A average continued to rise, peaking nine days after inoculation with a group average 1.7 °C higher than the pre-challenge mean (S.D. 0.6, see figure 2).

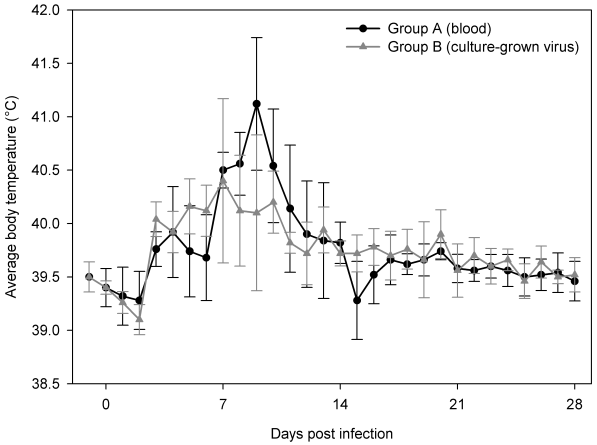

Figure 2: Average body temperatures of sheep after BTV-8 infection. The body temperatures of individual animals are available as supplemental material.

Overall, there was a total of 30 febrile days (individual body temperature over 40 °C) in group A and 26 days in group B. Mild clinical symptoms (increased nasal discharge, forced breathing, transient facial oedema) were recorded in both groups with no pronounced difference between the groups.
All sheep in group B and one sheep in group A were positive in the sandwich ELISA one week after infection. All sheep displayed BTV-specific antibodies in the next sample taken one week later (data not shown). One month after infection, virus neutralisation titres in group B (average [-log2] 6.6, S.D. 0.7) were higher than in group A (average [-log2] 5.6, S.D. 1.1) (data not shown).

All IFNAR-/- mice died within five days of injection of either culture-grown BTV or BTV in calf blood lysate. Survival times were similar for both inocula. All IFNAR-/- controls and C57BL/6 mice stayed healthy throughout the experiment (see table 1).

Group	Days post infection													
	0	1		2		3		4		5		6		7
Controls	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
BTV-8 in blood	100%	100%	100%	100%	100%	75%	0%	0%	0%	0%	0%	0%	0%	0%
BTV-8 supernatant	100%	100%	100%	100%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%
BTV-8 in blood 1:100	100%	100%	100%	100%	100%	100%	100%	50%	50%	0%	0%	0%	0%	0%
BTV-8 supernatant 1:100	100%	100%	100%	100%	100%	100%	100%	33%	33%	0%	0%	0%	0%	0%

Table 1: Survival times of mice after BTV-8 injection. Controls include C57BL/6 wild type mice injected with BTV and IFNAR-/- mice injected with BTV-negative blood or cell culture supernatant. The number of surviving mice at each time point is given as a percentage of the total number of inoculated mice in that group.

Discussion

Both the infectious blood lysate and culture-grown virus caused high-level viraemia in all sheep. The RT-qPCR results suggest a steadier course of infection in group B, but owing to the limited number of animals in the experiment, no robust statement can presently be made. Differences in the initial progression of viraemia can probably be attributed to the higher infectious dose in group B (culture-grown virus). Sheep in this group also seroconverted earlier and eventually reached higher titres of neutralising antibodies. Since the infectivity of freshly prepared blood lysate can only be determined retrospectively, it was not possible to use exactly matched challenge doses for both groups.

Conversely, sheep in group A (blood) displayed moderate pyrexia about a week after challenge, fulfilling the requirement of +1.7 °C laid out for controls in BTV challenge experiments by the World Organisation for Animal Health (OIE, 2009) while body temperatures of sheep in group B only rose mildly. An acute reaction to the bovine blood itself is unlikely due to the late onset of the temperature rise. This cannot be verified, however, because no control group of sheep injected with BTV-negative blood or supernatant was included in the experiment.

In any case, even the most prominent difference in average temperature between the groups (on day 9) was not statistically significant ($p = 0.0667$). The presumed low susceptibility of the breed (Eschbaumer et al., 2009) probably

contributed to the faint clinical manifestation in both groups. The environmental conditions in high-containment animal housing also play a role in the relatively mild BT symptoms often observed in experimentally infected ruminants (Verwoerd and Erasmus, 2004). On the other hand, both infectious blood and culture-grown BTV-8 invariably led to fatal infection in all IFNAR-/- mice. The limited number of mice available for the experiment, however, did not allow the determination of 50% lethal doses for the inocula.

To our knowledge, this is the first study directly comparing a BTV field strain that was only passaged in ruminants (as per OIE recommendations) to the homologous strain passaged in vitro. In other experiments that used both kinds of inoculum, infectious blood had merely been obtained by an animal passage of a virus isolate that had already been propagated in cell culture (MacLachlan et al., 1994; Martinelle et al., 2009).

In a challenge experiment, the most objective and reliable benchmark of BTV vaccine efficacy is the protection against viraemia (Savini et al., 2008), and regulating authorities consider it to be the main parameter (EMEA, 2008). Sterile immunity of vaccinated animals prevents vector-borne spread of the disease, and clinical BT in the absence of viraemia has never been reported. Accordingly, the reliable induction of viraemia in control animals is the most important feature of a suitable challenge model. The highly sensitive detection of BTV in blood samples by RT-qPCR is a well established, reproducible and reliable tool for monitoring BTV viraemia (Hoffmann et al., 2009). With less emphasis on clinical scoring, the use of culture-grown virus in challenge experiments should be reconsidered. The objective comparison of vaccine efficacy between different experiments depends on a well-defined inoculum of known infectivity, which is impractical with blood-derived inocula.

What is more, RT-PCR amplification of BTV genome fragments and sequencing using viral RNA from culture-grown virus is considerably easier than with viral RNA isolated from blood. This provides an additional means of ensuring the integrity of the inoculum and the absence of contamination with other serotypes.

But even though cell culture derived inocula have been used successfully in a number of recent challenge experiments (Boone et al., 2007; Di Emidio et al., 2004; Savini et al., 2009; Savini et al., 2007), the OIE currently discourages the use of virus isolates passaged in cell culture or embryonated chicken eggs (OIE, 2009).

In vitro and in ovo passages positively select for spontaneous mutations that promote replication in the heterologous culture system. This can lead to changes in virus phenotype, including reduced replication efficiency and virulence in the host (exploited in the production of live vaccines) as well as changes in tissue tropism (Alpar et al., 2009; Kirkland and Hawkes, 2004). But even highly

passaged, “attenuated” strains can cause viraemia sufficient for vector-borne spread (Batten et al., 2008; Ferrari et al., 2005; Listes et al., 2009) and disease (Veronesi et al., 2010). Interestingly, the ability to cross the placenta, the most prominent property acquired in culture passages of BTV, has repeatedly been reported for the European BTV-8 field strain (De Clercq et al., 2008; Worwa et al., 2009), suggesting that this strain might already have undergone culture adaptation (MacLachlan, 2009).

Conclusion

Culture-grown virus is easier to produce and standardise than infectious blood and less prone to deteriorate when stored for longer periods, and no additional animals are required for the production of challenge inoculum. In light of the similar outcome between the two different inocula this study found no compelling need for the use of blood in BTV-8 challenge experiments. A low number of cell culture passages of BTV-8 did not lead to attenuation compared to the field strain after a cattle passage. Both BTV-8 (Dal Pozzo et al., 2009; Darpel et al., 2007; Dungu et al., 2008) and other serotypes (Boone et al., 2007; Di Emidio et al., 2004; Savini et al., 2009; Savini et al., 2007) amplified in heterologous culture systems have been used successfully in many recent experiments. Furthermore, the protective efficacy of BTV vaccines can be adequately evaluated without recourse to clinical observations, which renders a challenge model that involves unnecessary suffering for the control animals ethically questionable. While the experimental induction of clinical disease is indispensable for pathogenesis studies, it is incidental in BTV vaccine efficacy trials. In general, the use of culture-grown virus allows a more standardised and rapid implementation of challenge studies, which is especially important considering the need for a swift response to the introduction of new BTV serotypes.

Conflict of interest

None of the authors has any financial or personal relationships with other people or organisations that could inappropriately influence this work.

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Re: Detection of bluetongue virus genome after vaccination with an inactivated vaccine

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Dear Editor:

In their recent article "Seroconversion, neutralising antibodies and protection in bluetongue serotype 8 vaccinated sheep"[1], C.A.L. Oura and colleagues mention in passing that they could not detect bluetongue virus (BTV) genome by real-time RT-PCR in blood samples taken after vaccination with an inactivated vaccine. Conversely, A. Steinrigl of the Austrian Agency for Health and Food Safety has reported in a presentation given at the 2009 annual meeting of the German Society for Virology[2] that his group found BTV genome in vaccinated sheep up to 61 days after vaccination.

Prompted by this presentation, the German national reference laboratory for bluetongue attempted to reproduce the results of the Austrian study. Four German Blackheaded Mutton yearling sheep were injected with a commercially available inactivated BTV serotype 8 vaccine (BTVPUR® AlSap 8, Merial S.A.S., Lyon, France; batch no. L242032) and ten EDTA blood samples for real-time RT-PCR analysis were taken over a period of seven days. In two sheep (nos. 1 and 2), the vaccine was applied subcutaneously into the lateral thoracic wall (as per manufacturer's instructions), while the other two sheep (nos. 3 and 4) were given the same amount of vaccine (1 ml) intravenously, diluted in 9 ml of sterile phosphate buffered saline (PBS) solution. The blood samples were analysed for BTV genome content using two different real-time RT-PCR assays[3,4].

RNA was extracted from 100 µl of each sample. Of a total of 100 µl of eluate, 5 µl were used for RT-PCR. BTV genome was never detected in the subcutaneously vaccinated sheep, but minute amounts were detectable for three days after intravenous injection of the vaccine (see table 1). At the low genome copy numbers encountered here, the generally excellent agreement of threshold cycle (C_t) values between the used assays breaks down due to the random distribution of individual RNA molecules among aliquots of the extracted sample. All samples were analysed in one real-time RT-PCR run, where both "no template" controls and all negative extraction controls gave no C_t.

Due to PCR inhibitors in the vaccine, we were unable to define a C_t value for RNA extracted directly from the injected vaccine preparation. However, linear real-time RT-PCR results were obtained for extractions from ten-fold dilutions of the inoculum, starting at 10^{-2} (C_t 25.13) down to 10^{-5} (C_t 35.15). Based on this, a C_t value of approximately 19 for the original vaccine could be deduced.

Assay	Route	Animal	Time after injection									
			-1 min	15 min	30 min	6 hrs	24 hrs	2 days	3 days	4 days	5 days	7 days
pan-BTV S5[3]	s.c.	Sheep 1	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
	s.c.	Sheep 2	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
	i.v.	Sheep 3	No C_t	35.39	35.40	34.87	38.45	No C_t	38.96	No C_t	No C_t	No C_t
	i.v.	Sheep 4	No C_t	39.00	35.29	40.40	37.75	39.21	No C_t	No C_t	No C_t	No C_t
BTV-8 VP2[4]	s.c.	Sheep 1	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
	s.c.	Sheep 2	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t	No C_t
	i.v.	Sheep 3	No C_t	37.52	36.53	36.09	No C_t	38.23	No C_t	No C_t	No C_t	No C_t
	i.v.	Sheep 4	No C_t	36.32	37.30	No C_t	No C_t	No C_t	37.54	No C_t	No C_t	No C_t

Table 1. Real-time RT-PCR results after vaccination of sheep with an inactivated BTV-8 vaccine.

Assuming a plasma volume of 2 litres[5] for a yearling sheep of 50 kg, the full dose of vaccine (1 ml) given intravenously will be diluted 2000-fold, hypothetically resulting in a C_t value of approximately 30 if distributed evenly. In our experiment, the C_t values were even higher than that, probably owing to inhomogenous distribution of the inoculum in the blood, and positive results were only obtained for a very limited time. In any case, intravenous injection of the full dose is an unlikely "worst-case" scenario. Poor vaccination technique could conceivably result in the introduction of small amounts of vaccine into blood vessels, but given the results of our experiment, it is doubtful whether this would be sufficient for detection by real-time RT-PCR.
Properly vaccinated sheep, on the other hand, where the vaccine is deposited under the skin, are not expected to score positive for BTV genome in peripheral blood at all, as is also confirmed by the results presented by Oura *et al.*[1].

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Real-time quantitative RT-PCR assays specifically detecting bluetongue virus serotypes 1, 6 and 8

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Abstract

Bluetongue virus (BTV) is a major pathogen of ruminants. Especially serotypes 1, 6 and 8 are of concern to veterinary authorities in Central Europe. This article describes highly sensitive real-time RT-PCR assays directed to BTV genome segment 2, for specific detection of BTV-1, -6 or -8 in animal samples.

Note

Bluetongue virus (BTV), an arthropod-borne *Orbivirus* in the family *Reoviridae* is a major pathogen of ruminants, and there are 24 established serotypes[18]. Until the end of the 20th century, bluetongue disease (BT) had been considered exotic to Europe. Since 1998, strains of at least five serotypes (BTV-1, -2, -4, -9 and -16) have been present along the Mediterranean coast[5,14]. In 2006, BTV-8 was first detected in the Netherlands without any previous notion of its presence in Europe[3]. Clinical disease in sheep was severe and, unusual for BTV, it was also seen in a wide range of non-ovine species including cattle, exacerbating the economic impact[5,13]. By the end of 2008, BTV-8 was present in all of Central Europe and beyond, and vaccination campaigns are ongoing[4,6,17]. At the same time, BTV-1 of Algerian origin has expanded northwards across the Iberian Peninsula and France[5], and in October 2008, BTV-6 was found in cattle in the Netherlands and later also in Germany[8,9]. Finally, in 2009, BTV-11 was reported in Flanders[10]. European Union monitoring requirements and the need for fast, reliable and sensitive detection of BTV RNA in animal samples led to the development of several real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays[7]. This article describes new assays specifically detecting either BTV-1, -6 or -8, to be used for further characterization of a BTV-positive sample.

For maximum specificity, our assays are directed to BTV genome segment 2 encoding the highly variable outer shell protein VP2[18]. VP2 sequences are available for at least one isolate of every established serotype[11] (NCBI GenBank). Several independent VP2 sequences have been published for BTV-1 and -8[12]. Among these, we gave priority to sequences of European isolates. The sequence of segment 2 of “NET2006/04”, the European reference isolate of BTV-8, was found to have higher similarity to a 1982 isolate from Nigeria than to the South African BTV-8 reference strain[12]. On the other hand, segments 2 of North African and French isolates of BTV-1 are close to the reference strain “RSArrrr/01”, all belonging to the “western group”[16]. For BTV-6, VP2 of the recently emerged strain is over 99% identical to the South African BTV-6 reference strain “RSArrrr/06”[15], the only published sequence. Accordingly, primers and hydrolysis probes (see table 1) were selected using “Beacon

Designer" (PremierBiosoft International, USA), and "primer3" design software (http://frodo.wi.mit.edu/). The probes were labeled with 6-carboxyfluorescein (6-FAM) at the 5' end, and "black hole quencher-1" carboxylic acid (BHQ-1) was attached to the 3' end. Oligonucleotides were synthesized by a commercial supplier (Eurogentec Deutschland GmbH, Germany).

BTV1-VP2-Mix1-FAM	(product size 123 bp)	
Type	Name	Sequence
Forward primer	BTV1-VP2-186F	5' CGG ACC GCA TTA TGG TAT AAC C 3'
Reverse primer	BTV1-VP2-308R	5' ACT CTT GTG TCT CGT ACT TTC AAC 3'
Probe	BTV1-VP2-203FAM	5' ACC GCC CGT CTT TCA TCG TAA CCC 3'

BTV1-VP2-Mix2-FAM	(product size 120 bp)	
Type	Name	Sequence
Forward primer	BTV1-VP2-2407F	5' CCT CAA AGG CGA TTC GAT TTA GC 3'
Reverse primer	BTV1-VP2-2526R	5' TCA CGA CGT TGT AGT TGA CTC C 3'
Probe	BTV1-VP2-2438FAM	5' TGA AGC GCA GCC CAA GAT TGC ACG 3'

BTV6-VP2-Mix1-FAM	(product size 97 bp)	
Type	Name	Sequence
Forward primer	BTV6-VP2-785F	5' GAT ACG TGA TGC GTG GAT TG 3'
Reverse primer	BTV6-VP2-881R	5' TAC CAC CTT CCT TCC GAC AC 3'
Probe	BTV6-VP2-817FAM	5' ATC CGA GGC ATA TTC GCT CGC TGG 3'

BTV6-VP2-Mix2-FAM	(product size 89 bp)	
Type	Name	Sequence
Forward primer	BTV6-VP2-1056F	5' TAT AAT GGC AGA ATA TGG TGG AC 3'
Reverse primer	BTV6-VP2-1144R	5' CAG TAA ACA TCG CCC AAC CT 3'
Probe	BTV6-VP2-1081FAM	5' ATC CGT ACC CTT GCT TGC GTG GAG 3'

BTV8-VP2-Mix1-FAM	(product size 86 bp)	
Type	Name	Sequence
Forward primer	BTV8-VP2-1604F	5' GTT ACG CAT TAC CGA GGT TGT G 3'
Reverse primer	BTV8-VP2-1689R	5' GAT CAT GTG TGA ACG CCT TCG 3'
Probe	BTV8-VP2-1631FAM	5' AAC GGC TCA CAC CGA CGA TCC AGC 3'

Table 1. Primers and probes.

Before one-step RT-PCR could be performed, double-stranded RNA extracted from samples needed to be denatured. Briefly, template RNA was dispensed onto PCR plates; these were sealed, heated for 5 minutes at 95 °C, snap-frozen in liquid nitrogen and placed in a cooling rack at -20 °C. RT-PCR master mix was added to the plate, the plate was resealed and centrifuged briefly before placement in the thermal cycler.

Two commercially available kits were used for amplification, iScript One-Step RT-PCR Kit for Probes (Bio-Rad Laboratories, USA), and QuantiTect Probe RT-PCR Kit (QIAGEN, Germany). For the iScript kit the RT-PCR master mix consists of 5 µl of nuclease-free water, 12.5 µl of 2-fold reaction mix, 0.5 µl of reverse transcriptase and 2 µl of custom primer/probe mix per reaction. Primer/probe mixes contain forward and reverse primers at a concentration of 10 pmol per µl and labeled probe at 1.25 pmol per µl in 0.1-fold TE buffer. 20 µl of chilled master mix are added to 5 µl of snap-frozen denatured template RNA for one-step RT-PCR. Validation data presented herein was collected using the iScript kit (see figure 1). The QuantiTect kit produced very similar results (data not shown). A reference sample containing BTV RNA of the appropriate serotype was serially diluted ten-fold in RNA-safe buffer containing 50 ng per µl poly-A carrier RNA, 0.05% Tween 20 and 0.05% sodium azide in nuclease-free water. One-step RT-PCR was carried out for 10 minutes at 50 °C and 5 min at 95 °C followed by 42 cycles of 15 seconds at 95 °C, 20 sec at 56 °C and 30 sec at 72 °C in an Mx3005P QPCR system (Stratagene, USA).

There was no cross-reactivity with BTV field isolates or reference strains (obtained from the European community reference laboratory for BT) of the heterologous serotypes for all three serotype-specific RT-qPCR assays, demonstrating an analytical specificity[2] of 100%. No cross-reaction was observed with isolates of epizootic hemorrhagic disease virus of different serotypes (data not shown).

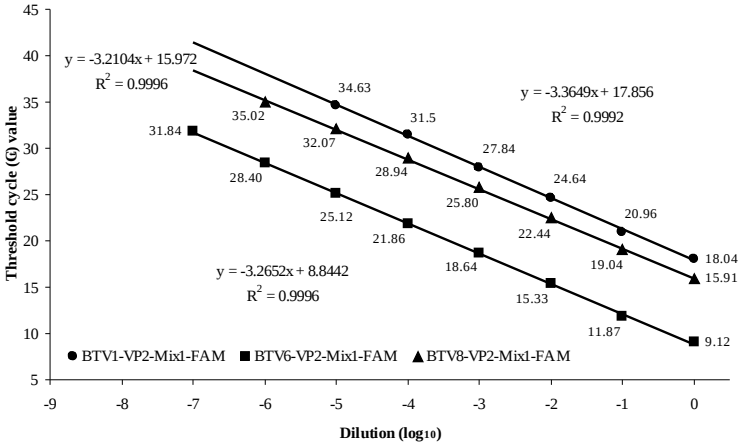

Figure 1. Real-time RT-PCR results for serial dilutions of RNA of BTV-1, -6 and -8 using the appropriate primer/probe mixes. For BTV-1 and BTV-6, the alternative mixes produced similar results (BTV1-VP2-Mix2-FAM: y = -3.4471x + 17.383, R2 = 0.9983 and BTV6-VP2-Mix2-FAM: y = -3.2771x + 8.5275, R2 = 0.999; data not shown).

For clinical samples submitted to the German national reference laboratory (NRL) for BT and for samples from in-house animal experiments, the assays proved to be as sensitive as the highly sensitive "pan-BTV" assay currently used at our laboratory[1,19] (see table S1 in the supplemental material). For selected samples, serotype identification was confirmed by sequencing of the amplicons (data not shown). The equivalent analytical sensitivity of the used RT-qPCR assays leads to highly similar results between them. In routine diagnostics at the NRL, the serotype-specific assays generally had a clinical sensitivity[2] of 100% for samples that had a threshold cycle (C_t) value of less than 36 in the "pan-BTV" assay (data not shown). Very low viral genome loads can lead to inconsistencies between assays or between replicates of the same assay, owing to the random distribution of individual molecules of viral RNA among aliquots of total RNA extracted from a sample.

Sample	pan-BTV S5 C_t value	BTV-8 VP2 C_t value	BTV-6 VP2 mix 1 C_t value	BTV-6 VP2 mix 2 C_t value	BTV-1 VP2 mix 1 C_t value	BTV-1 VP2 mix 2 C_t value
subm. #308-00, 2008	27.82	No C_t	No C_t	No C_t	27.54	27.40
subm. #008-01, 2009	28.69	No C_t	No C_t	No C_t	29.10	28.60
subm. #060-01, 2009	28.04	No C_t	No C_t	No C_t	29.52	28.80
exp. 3/08 anim. 503, 03 dpi	34.92	No C_t	No C_t	No C_t	35.12	33.82
exp. 3/08 anim. 503, 09 dpi	29.93	No C_t	No C_t	No C_t	29.69	29.51
exp. 3/08 anim. 503, 18 dpi	33.49	No C_t	No C_t	No C_t	34.64	33.88
exp. 3/08 anim. 764, 03 dpi	36.34	No C_t	No C_t	No C_t	37.10	35.80
exp. 3/08 anim. 764, 09 dpi	24.43	No C_t	No C_t	No C_t	24.78	24.71
exp. 3/08 anim. 764, 18 dpi	29.25	No C_t	No C_t	No C_t	29.50	30.02
supernatant BTV-1 FR/07	22.89	No C_t	No C_t	No C_t	25.83	25.48
subm. #201-04, 2008	29.76	No C_t	27.60	28.85	No Ct	No Ct
subm. #268-04, 2008	29.26	No C_t	27.90	28.29	No Ct	No Ct
subm. #290-01, 2008	32.24	No C_t	30.18	30.72	No Ct	No Ct
subm. #295-03, 2008	32.27	No C_t	33.90	31.10	No Ct	No Ct
subm. #295-06, 2008	31.31	No C_t	33.31	30.93	No Ct	No Ct
subm. #300-01, 2008	29.01	No C_t	27.12	28.35	No Ct	No Ct
subm. #300-02, 2008	28.78	No C_t	26.35	26.52	No Ct	No Ct
subm. #077-00, 2009	28.52	No C_t	27.80	28.19	No Ct	No Ct
supernatant BTV-6 DE/08	25.33	No C_t	27.43	26.71	No Ct	No Ct
subm. #161-01, 2008	29.86	28.48	No C_t	No C_t	No C_t	No C_t
subm. #196-01, 2008	28.43	27.00	No C_t	No C_t	No C_t	No C_t
subm. #282-02, 2008	29.71	28.99	No C_t	No C_t	No C_t	No C_t
subm. #284-11, 2008	26.75	25.23	No C_t	No C_t	No C_t	No C_t
subm. #311-02, 2008	30.38	29.88	No C_t	No C_t	No C_t	No C_t
subm. #097-01, 2008	22.07	23.57	No C_t	No C_t	No C_t	No C_t
subm. #023-00, 2009	39.12	40.43	No C_t	No C_t	No C_t	No C_t
supernatant BTV-8 DE/08	23.69	24.77	No C_t	No C_t	No C_t	No C_t

Table S1. Real-time qRT-PCR results for clinical samples submitted to the NRL and samples from animal experiments.

The serotype-8-specific assay does not detect the South African BTV-8 reference strain “RSArrrr/08”. This is intentional and a consequence of the tailoring of the assay to the European isolates. The sudden appearance of a genetically different strain of BTV-8 would signify a new introduction event. Detecting this in routine diagnostics requires an assay with strict specificity for the local strain. At the same time, this also allows the detection of emerging nucleotide variations (antigen drift) in the local strain. In any case, the initial evaluation of a sample should be done with at least one robust group-specific “pan-BTV” assay, and if the serotype cannot be readily identified with the described assays, the sample must be further analyzed by an alternative method.
In conclusion, all RT-qPCR assays presented here reliably detect bluetongue virus with high sensitivity and serotype specificity. Each assay specifically detects one serotype with no cross-reactivity. With two independent assays each for serotypes 1 and 6, it is possible to cross-confirm positive results. All assays are used successfully for routine diagnostics at the German NRL for BT. From January 2008 to March 2009, over 10,000 submitted samples were tested for the presence of BTV genome. Of these, 1,303 were screened for BTV-8 (880 positives), 937 also for BTV-6 (88 positives) and 142 for BTV-1 (four positives). To our knowledge, no false positives have ever been identified, and all tested assays had a diagnostic specificity of 100%. The serotype-specific

assays had already been made available to regional veterinary laboratories in Germany, and both the BTV-6 and BTV-8-specific assays have since been evaluated successfully in ring trials.
Studies assessing the feasibility of multiplex assays directly differentiating between BTV serotypes are underway.

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Emergence of bluetongue virus serotype 6 in Europe – German field data and experimental infection of cattle

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Abstract

In late 2008, bluetongue virus (BTV) serotype 6 (BTV-6), which had never occurred in Europe before, was first detected in the Netherlands and Germany. While the origin of the virus remains unknown, the prevalence of infections in cattle was investigated in a virological (N= 28,658) and serological (N= 2,075) field survey in Lower Saxony, where 45 cases confined to the district Grafschaft Bentheim were found. Blood from affected animals was used for the experimental infection of three cattle with different BTV antibody status, leading to sustained viraemia in one animal naïve for BTV. Of two animals that had detectable antibodies against BTV serotype 8, one became transiently infected and seroconverted for BTV-6 while the other did not react. In conclusion, while only a very limited spread of BTV-6 could be observed in the field, experimental infection of cattle did not show substantial differences of the course of infection in comparison to other BTV serotypes.

Introduction

Bluetongue in Europe

Bluetongue (BT) is an arthropod-borne disease of ruminants and camelids caused by any of 24 established serotypes of Bluetongue virus (BTV), an Orbivirus of the family Reoviridae. It is principally transmitted by haematophagous Culicoides midges (Schwartz-Cornil et al., 2008). Bluetongue virus did not regularly occur in mainland Europe before 1998. Since then, serotypes 1, 2, 4, 9 and 16 have been circulating in Southern Europe (Saegerman et al., 2008). In recent years, a BTV-1 strain of Algerian origin has expanded northwards across the Iberian Peninsula and France and its intrusion into the Benelux and Germany appears imminent (Hateley, 2009). After its first detection in the Netherlands in 2006, BTV-8 has spread across Central Europe and beyond in three seasons, eventually reaching as far as Norway and Israel. Vaccination campaigns against BTV-1 and -8 are ongoing. In the beginning of 2008, a putative new BTV serotype, “Toggenburg orbivirus”, has been detected in goats in Switzerland (Hofmann et al., 2008) and later that year, BTV serotypes 6 and 11 (ISID ProMED-mail, 2008b; ISID ProMED-mail, 2009) appeared in Europe for the first time.

BTV-6 in the Netherlands and Germany

In October 2008, BTV was detected in the Netherlands in animals previously vaccinated against BTV-8. Routine sequencing of real-time RT-PCR amplicons yielded only 95% nucleotide identity of genome segment 10 to the European strain of BTV-8, and the introduction of a new strain or serotype was suspected by the Dutch national reference laboratory for bluetongue (NRL BT) at the Central Veterinary Institute of Wageningen UR (CVI), Lelystad (ISID ProMED-mail, 2008a).
The European Community reference laboratory (CRL) at the Institute for Animal Health, Pirbright, UK, was able to isolate the virus from a Dutch sample and identified it as BTV-6, with over 99.9% segment 2 (VP2) sequence identity to the South African modified-live virus (MLV) vaccine strain (ISID ProMED-mail, 2008b-c). Vector-borne spread of vaccine viruses has been reported before (Savini et al., 2008; Listeš et al., 2009). A BTV-6 restriction zone was created, entailing a mandatory screening of all animals to be moved outside the zone.
The eastern part of the restriction zone covered German territory. Here, the first case of BTV-6 was confirmed by the NRL BT at the Friedrich-Loeffler-Institut (FLI), Insel Riems, on November 5, 2008.
Subsequently, a field survey was undertaken to determine the virological and serological prevalence of BTV-6 in the affected area. At the NRL, initial attempts to isolate BTV-6 from submitted samples in cell culture and embryonated chicken eggs did not succeed. Since inoculation of susceptible animals is considered the most sensitive method (Clavijo et al., 1999), an animal experiment was conducted to obtain a virus isolate and investigate the virulence of BTV-6 in cattle.

Materials and Methods

Field survey

Initially, infection with BTV-6 was detected in four cattle in three holdings in the district Grafschaft Bentheim, Lower Saxony, Germany. Further samples were taken from these animals in December 2008 and January 2009 and epidemiological inquiries were performed in the holdings by epidemiologists of the FLI.
Regional laboratories in Lower Saxony conducted a survey to study the spatial distribution of the infection. The majority of samples were taken along the Dutch border and around the initially affected holdings, where the risk of exposure to BTV-6 infected midges was highest. In total, 28,658 animals were investigated using the “pan-BTV” group-specific real-time RT-PCR assay by

Toussaint et al. (2007). Initially, samples from BTV-positive animals were then evaluated at the NRL with real-time RT-PCR assays specific for BTV-6 and -8 (Hoffmann et al., 2009b). For ongoing surveillance, these assays were outsourced to the regional laboratories in late November.
Serum samples (N= 2,075) from all susceptible animals in the first three affected holdings and all holdings within a radius of 1 kilometre were sent to the bluetongue reference laboratory of the World Organisation for Animal Health (OIE) at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" (IZSAM), Teramo, Italy, for BTV neutralisation assays (Gard and Kirkland, 1999).

Animal experiment

Animals and experimental design

Three male Holstein Frisian calves were obtained locally and transferred to the BSL-3 facility of the FLI on Insel Riems, where they were confirmed to be free of circulating BTV genome by real-time RT-PCR analysis of blood samples (Toussaint et al., 2007). They came from a region where BTV has never been reported. They were purchased as immunised against BTV-8 with two doses of an inactivated vaccine (BTVPUR® AlSap 8, Merial S.A.S., Lyon, France) but displayed different levels of BTV-specific antibodies (Table I). The calves had been vaccinated with a gE-deleted BHV-1 marker vaccine. They had detectable antibodies against BVDV, and were negative for BVDV antigen.

Animal	Assay	Day 0	Day 7	Day 14	Day 21	Day 60
Calf 588	DR ELISA	8	8	16	2048	1024
	NA BTV-6	0	0	0	10	100
	NA BTV-8	4	3	3	2	0
Calf 605	DR ELISA	0	0	32	128	64
	NA BTV-6	0	0	3	40	320
	NA BTV-8	0	0	0	0	0
Calf 607	DR ELISA	64	64	64	64	32
	NA BTV-6	0	0	0	0	0
	NA BTV-8	8	6	4	4	3

Table I: Serological data from the animal experiment. Values given for virus neutralisation assays (NA) are 50% neutralising doses against the indicated serotype, double-recognition (DR) ELISA values denote the reciprocal of the last serum dilution that gave a positive result.

On day 0 of the study, the animals were inoculated intravenously and subcutaneously (multiple sites in the shoulder region) with BTV-6 positive blood pooled from samples submitted to the NRL. None of the samples contained detectable BTV-8 RNA (data not shown). Packed blood cells of the inoculum had been repeatedly washed with phosphate-buffered saline solution (PBS) and then ultrasonically disrupted. The blood preparation was tested free of bacterial contamination and it had a threshold cycle (C_t) value of 26.87 in the BTV-6 specific real-time RT-PCR (Hoffmann et al., 2009b).
After inoculation, blood samples were taken at regular intervals. Blood was drawn by jugular puncture and collected in tubes containing potassium EDTA or clot activator, respectively (Monovette, Sarstedt, Nümbrecht, Germany). Samples were stored at 4 °C until analysis. During the entire study, rectal body temperatures were taken daily, and the calves were monitored for clinical signs.

Serology

Serological data were collected from samples taken on days 0, 7, 14, 21 and 60 after infection, using a commercially available double-recognition (DR) ELISA (PrioCHECK® BTV DR, Prionics Deutschland GmbH, Planegg-Martinsried, Germany) to detect BTV group-specific antibodies against the highly conserved core protein VP7. Virus neutralisation assays against German isolates of BTV-8 and -6 were performed as described previously (Eschbaumer et al., 2009). The BTV-6 isolate was obtained by cell culture inoculation with blood taken from calf 605 on day 7 post-infection (dpi) and subsequently passaged on Vero cells

(RIE15, Collection of Cell Lines in Veterinary Medicine [CCLV], FLI, Insel Riems, Germany).

Detection of viral RNA and virus isolation

Total RNA from whole blood samples was extracted manually using a commercial kit (QIAamp Viral RNA Mini Kit, QIAGEN, Hilden, Germany). The amount of BTV genome in the samples was determined using the "pan-BTV" assay by Toussaint et al. (2007) and the in-house BTV-6 and BTV-8 assays that had also been used for the field samples (Hoffmann et al., 2009b). The "pan-BTV" assay amplifies a highly conserved sequence at the 5' end of BTV genome segment 5, while the serotype-specific assays are directed to genome segment 2, encoding the outer shell protein VP2. One-step RT-PCR was carried out using a commercial kit (iScript One-Step RT-PCR Kit for Probes, Bio-Rad Laboratories, Hercules, CA, USA) in an Mx3005P QPCR system (Stratagene, La Jolla, CA, USA).
For RT-PCR-positive samples, virus isolation was performed by a method adapted from Clavijo et al. (1999), using embryonated chicken eggs as well as Vero (RIE15, CCLV) and baby hamster kidney (BHK) 21 clone 13 cells (RIE179, CCLV). Briefly, blood cells were packed, washed repeatedly with PBS and ultrasonically disrupted on ice. Intravenous inoculation of 10-day-old chicken embryos and adsorption onto 75% confluent cell layers was performed in parallel. After 5 days of incubation, embryo organ lysate and cell culture supernatants were blindly passaged in cell culture. When a cytopathic effect developed in the second passage, BTV-6 replication was confirmed by serotype-specific real-time RT-PCR.

Results

Epidemiological investigations and field survey

All three initially affected holdings were dairy farms whose animals had not been in contact with other cattle or sheep either on the farm or on pasture. Two farms had their own breeding stock, and the third had only bought animals from Lower Saxony in 2008. Animals on the farms were healthy and well kept. Record-keeping was complete and consistent, and there was no indication of the illegal use of modified-live bluetongue vaccines. The distance to the closest affected holding in the Netherlands was found to be only 17 kilometres, while the farthest was 52 kilometres away. No direct epidemiological link between the farms could be established.

Of the 28,658 animals in Lower Saxony that were tested by regional laboratories, 3,753 (12.71-13.49%, 95% confidence interval) were positive in the group-specific "pan-BTV" real-time RT-PCR. Among them, 45 BTV-6 positive animals (overall prevalence 0.11-0.21%) in 23 holdings (Figure 1) were found. In at least two cases, BTV-6 and BTV-8 genome was detected in the same animal by serotype-specific real-time RT-PCR analysis (data not shown). For 36 BTV-6 positive animals identified by the NRL in November 2008, C_t values ranged from 25.56 to 34.72 (average 30.38). Table II shows the results of the longitudinal virological monitoring of the first four cases together with serological data from the first sampling in November. The amount of viral genome detected in these animals slowly decreased within the survey period of 10 weeks. All four animals were negative in the BTV-8 specific real-time RT-PCR.

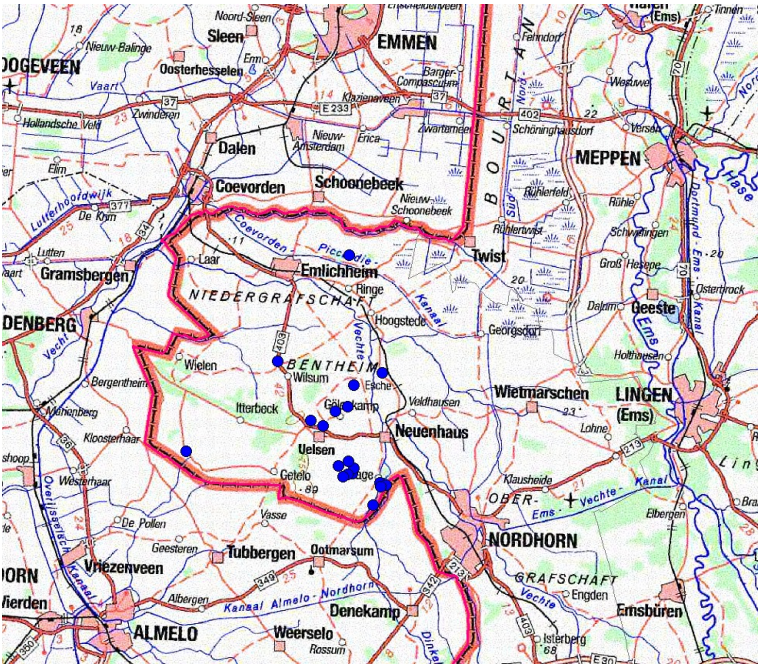
Animal	Virus neutralisation		BTV-6 real-time RT-PCR		
	BTV-6	BTV-8	5 Nov 08	8 Dec 08	22 Jan 09
1328	40	0	C_t 26.86	C_t 26.83	C_t 29.00
1338	40	0	C_t 26.82	C_t 27.00	C_t 30.46
2635	40	160	C_t 30.66	C_t 29.36	C_t 30.14
9707	40	40	C_t 29.85	C_t 27.65	C_t 29.23

Table II: Two-month follow-up on the first four BTV-6 positive animals. Threshold cycle (C_t) values given were obtained with a real-time RT-PCR assay specific for BTV-6. Values for neutralisation assays (NA) are 50% neutralising doses for the indicated serotype.

Based on data from the German animal identification and registration system (HI-Tier; Kroschewski et al., 2006), BTV-8 vaccine coverage in the district Grafschaft Bentheim was 96% for female cattle, 69% for sheep and 47% for goats. In the 2,075 sera tested at IZSAM, only antibodies against BTV-8 and -6 were found (Table III), while all samples were negative for serotypes 1, 2, 4, 9, 15 and 16. Neutralising antibodies against BTV-8 were detected in 1,218 (56.55-60.83%) cases and BTV-6-specific antibodies in 39 animals (1.34-2.56%), 27 of which also had neutralising antibodies against BTV-8 (0.86-1.89%). Neutralising titres against BTV-8 ranged from 1:10 to over 1:1280, BTV-6 titres were between 1:10 and 1:640.

Sample origin	No. of samples	BTV-8 pos.	BTV-6 pos.	Both
First 3 holdings	858	574	23	15
1 km zone	1217	644	16	12
Total:	2075	1218	39	27
		(56.55-60.83%)	(1.34-2.56%)	(0.86-1.89%)

Table III: Results of BTV neutralisation assays for field samples. No neutralising antibodies against serotypes 1, 2, 4, 9, 15 or 16 were detected in any sample. Percentages are given as a 95% confidence interval.



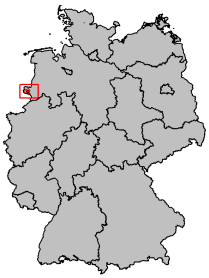


Figure 1. Cattle herds with BTV-6 infections in Germany. Dots (blue) mark the locations of affected holdings. The thick line (red) is the Dutch-German border.

Animal experiment

While the calves had reportedly been vaccinated against BTV-8 in mid-2008 with two doses of an inactivated vaccine, their serological status was diverse (Table I). After inoculation with BTV-6, two animals (calves 605 and 588) displayed measurable virus replication (as detected by real-time RT-PCR, Table IV) and subsequently seroconverted for BTV-6 after two and three weeks, respectively, together with a marked increase in group-specific antibodies (Table I). Calf 607 failed to react both in the real-time RT-PCR (data not shown) and by serology.
Calf 588 was only transiently infected and had already fully cleared the virus four weeks after infection, but the viraemia in calf 605 lasted over six weeks (Table IV). The animal remained BTV-6-positive in the real-time RT-PCR for over 100 days until the end of the study period. BTV-8 was not detected in any animal (data not shown).

Day	Calf 588			Calf 605		
	pan-BTV real-time	BTV-6 real-time	virus isolation	pan-BTV real-time	BTV-6 real-time	virus isolation
0	No C_t	No C_t	ND	No C_t	No C_t	ND
5	No C_t	No C_t	ND	No C_t	No C_t	ND
7	No C_t	No C_t	ND	29.41	28.26	positive
8	No C_t	No C_t	ND	27.44	26.86	ND
9	35.62	32.23	negative	27.03	26.43	ND
10	32.81	31.14	negative	26.03	25.29	ND
12	No C_t	34.35	negative	26.53	28.02	positive
13	33.28	32.22	negative	23.66	21.36	ND
14	32.75	31.94	negative	22.79	22.00	ND
17	32.95	32.56	positive	26.39	23.95	ND
19	33.76	34.04	negative	26.04	24.81	positive
21	35.78	No C_t	negative	27.85	26.93	positive
24	No C_t	No C_t	ND	29.06	27.94	positive
27	38.23	No C_t	ND	30.11	29.35	positive
31	No C_t	No C_t	ND	29.92	29.35	positive
34	No C_t	No C_t	ND	30.97	29.03	positive
38	No C_t	No C_t	ND	31.10	29.90	positive
45	No C_t	No C_t	ND	30.29	29.40	positive
52	No C_t	No C_t	ND	32.74	29.93	negative
60	No C_t	No C_t	ND	30.82	27.84	negative
69	No C_t	No C_t	ND	29.55	27.88	negative
77	No C_t	No C_t	ND	30.66	29.90	ND
84	No C_t	No C_t	ND	31.30	30.69	ND
91				32.06	32.15	ND
98				31.32	30.56	ND
105				31.83	31.18	ND

Table IV: Real-time RT-PCR and virus isolation from samples from the animal experiment

Threshold cycle (C_t) values and results of the second passage of the virus isolation are given where applicable, ND = not determined. No BTV-8 genome was detected in any animal and no BTV-6 genome was detected in calf 607 (data not shown).

Between days 5 and 9 after infection, calf 605 had a slightly elevated body temperature and calf 588 showed a similar increase in temperature from day 10 to 12 (data not shown), while no other clinical signs could be observed.

Discussion

Among tens of thousands of animals that were screened in the field survey, only a small number was found to be positive for BTV-6 RNA or antibodies. These were mostly isolated cases in holdings scattered throughout a single district in the German federal state of Lower Saxony, close to the border with the Netherlands, where similar findings were made (MinLNV, 2009a). Given the BTV screening measures that were in place in 2008, BTV-6 most likely was detected much earlier after its introduction than BTV-8 had been, suggesting an entry late in the season. Apparently it has only been able to propagate to a very limited extent, while BTV-8 had spread rapidly after its introduction in 2006 (Hateley, 2009). Together with the reported genetic similarities, this gave rise to the theory that BTV-6 was an attenuated strain introduced either by the illegal local use of a modified live vaccine (ISID ProMED-mail, 2008d) or the unrecognized import of animals that had been vaccinated with a modified live vaccine in a third country and were still viraemic for BTV-6 when they arrived in Europe (MinLNV, 2009a).
Serological evidence from areas affected by BTV-6 provided no indication of a recent use of the commercially available multivalent MLV vaccine. No antibodies against serotypes other than BTV-6 and -8 were detected in either the Netherlands (MinLNV, 2009a) or Germany (this study). Specifically, there were no traces of other serotypes from bottle A of the multivalent vaccine package (1, 4, 12 and 14) (Dungu et al., 2004), which would have been the most likely source of the BTV-6 vaccine strain. This however has no bearing on another possible explanation, the accidental introduction of vectors already infected with BTV-6 as stowaways in global trade (Mintiens et al., 2008).
According to the CRL, genome segment 2 of the Netherlands isolate is almost identical to the South African BTV-6 vaccine strain (ISID ProMED-mail, 2008d). Whether this isolated finding is particularly meaningful remains open to debate. The manufacturer of the vaccine (Onderstepoort Biological Products, Onderstepoort, South Africa) maintains that based on segment 2 alone, all African strains of BTV-6, including the vaccine, are closely related (ISID

ProMED-mail, 2008e). Such a high degree of homology was not seen in European BTV-8, however, which shares 93% of nucleotides of segment 2 with the South African BTV-8 vaccine strain, and 97% with a Nigerian isolate (Maan et al., 2008). For other segments of European BTV-6, a high similarity to the South African reference strain is claimed, but the origin of segment 10 is unknown (MinLNV, 2009b) and reassortment has been suspected (Saegerman and Pastoret, 2009). Only four isolated sequences of individual genome segments of BTV-6 are presently available online, and no sequence of either the BTV-6 vaccine strain or the recent European isolates has been made public (U.S. National Center for Biotechnology Information, GenBank, http://www.ncbi.nlm.nih.gov/ Genbank/).

But regardless of its origin, the question remains if the very limited spread of BTV-6 that has been observed in 2008 is due to attenuation of the virus itself. Judging from our data, other factors could have contributed to that outcome. Our experiment suggests that the susceptibility to BTV-6 infection of cattle is influenced by previous exposure to BTV-8, but this needs to be investigated in a broader study using a defined challenge dose of BTV-6. In the present experiment, it is difficult to estimate the infectious dose contained in the inoculum. The dates of infection of the donor animals remain unknown, but the low amount of BTV-6 genome in the pooled samples (no C_t value lower than 25, data not shown) and the failure to isolate virus by other means suggest that the blood had been collected at a late stage of infection, resulting in limited infectivity. While BTV-infected ruminants can remain positive in the RT-PCR and even viraemic for weeks or months, the concentration of infectious particles in the blood decreases over time (Singer et al., 2001; MacLachlan et al., 2009; Hoffmann et al., 2009a). In a related animal experiment at the CVI, using a BTV-6 field sample from a Dutch cow, at first only one of six animals could be infected, which also suggests a low infectious dose. When fresh blood taken from the latter animal at 10 dpi was inoculated in a second experiment, however, 11 out of 11 animals displayed virus replication (Van Rijn, 2009).

Nevertheless, within the scope of our experiment, the course of BTV-6 infection once established in the naïve calf was similar to BTV-8. No data are available for the BTV-6 MLV vaccine strain, but the longest duration of viraemia reported after vaccination of cattle with other MLV vaccines was 28 days (Monaco et al., 2004) – less than two thirds of the viraemic period in calf 605. Long-lasting viraemia has been observed with an insufficiently attenuated strain of BTV-16 (Savini et al., 2008), and up to 63 days of viraemia have been described after experimental infection of cattle with virulent BTV strains (Singer et al., 2001; Dal Pozzo et al., 2009). On the other hand, the high number of culture passages commonly used in the production of MLV vaccines (Savini

et al., 2008) might actually facilitate the subsequent reisolation of vaccine viruses in vitro.

At its peak, the amount of BTV-6 genome detected in calf 605 did not notably differ from viral genome levels observed in BTV-8 animal experiments (Darpel et al., 2007). This similarity also extends to the persistence of detectable viral RNA in peripheral blood of calf 605 and the only gradual decrease of circulating BTV-6 genome in the presence of neutralising antibodies that was observed in field-infected animals (Table II) (Dal Pozzo et al., 2009). Viral RNA remains detectable in the blood for extended periods of time because BTV persists in invaginations in the cell membrane of erythrocytes (Schwartz-Cornil et al., 2008), a trait probably shared by both field and vaccine strains.

With regard to the absence of any clinical symptoms other than elevated body temperature, it has to be noted that in our experience and based on experimental data reported by other groups (Darpel et al., 2007), clinical bluetongue disease in cattle does occur, but it is not a regular finding in Holstein Frisians experimentally infected with BTV-8 either. Conversely, it has been shown that even vaccine strains can cause disease (Savini et al., 2008), if only in sheep (Veronesi et al., 2005). Exposure to sunlight might play a role in bluetongue pathogenesis, which could contribute to the reduced severity of disease observed after experimental infection in high-containment animal housing (Verwoerd and Erasmus, 2004). The initial detection of BTV-6 in the Netherlands, on the other hand, had been precipitated by clinical disease observed in the field (ISID ProMED-mail, 2008d; Hateley, 2009).

There is no doubt that after two years of the BTV-8 epidemic and with mass vaccinations beginning in mid-2008, BTV-6 faced by no means an animal population naïve to BTV (Saegerman and Pastoret, 2009). To the contrary, the western North European Plain shared by Belgium, the Netherlands and Germany arguably was the region with the highest cumulative incidence of BTV-8 in Europe. In the field survey presented here, over half of the animals had neutralising antibodies to BTV-8, and more than one tenth were still positive in the real-time RT-PCR. Even if cross-neutralisation between serotypes is low, there is evidence of group-specific cell-mediated immunity after infection (Schwartz-Cornil et al., 2008). Given these unfavourable conditions, the fact that BTV-6 was able to spread at all does not suggest attenuation.

Conclusion

The final evaluation as well as the measures taken by animal health authorities concerning the emergence of BTV-6 (SCoFCAH, 2009) are debatable from a scientific point of view (Saegerman and Pastoret, 2009). If BTV-6 does not return in 2009, it will be difficult to say with any certainty whether this was because of its vaccine origin or simply because BTV-8 had come first.
In either case, one troubling fact remains: since 2006, three different serotypes of BTV have first been detected in the same geographical region, without any previous notion of their presence on European soil and without any definite explanation for their introduction (Mintiens et al., 2008; MacLachlan et al., 2009). Finding and closing that loophole is crucial for animal health in Europe, particularly with regard to the looming threat of other, more lethal, orbivirus diseases of domestic animals like African horse sickness.

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European Bluetongue virus serotypes 6 and 8: Studies on virulence and cross-protection

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European Bluetongue virus serotypes 6 and 8: Studies on virulence and cross-protection

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Bluetongue virus (BTV), an arthropod-borne *Orbivirus* (family *Reoviridae*) is a global pathogen of ruminants. There are 24 established serotypes.
BTV had never occurred in Central Europe until the introduction of serotype 8 (BTV-8) in 2006[1]. By 2008, it had spread as far as Norway, Spain and Hungary, infecting over 25,000 farms in Germany alone[2]. Later that year, another serotype (BTV-6) was detected in the eastern part of the Netherlands and northwestern Germany. It was shown to be very closely related to a modified-live vaccine (MLV) strain by genetic analysis[3].

Fig. 1: Map of BTV restriction zones as of 7 Nov 2008[19]. Red: BTV-8, yellow: BTV-6.

In a field survey, the prevalence of BTV-6 in the region was found to be extremely low and no clinical disease was recorded[4]. It did not spread any further in 2009. Conversely, other MLV strains had been shown to be pathogenic for sheep[5-6], and have been widely spread by vectors[7-8].

This study compares the virulence of European BTV-6 and BTV-8. It further investigates if previous BTV-8 infection or vaccination has an effect on BTV-6 infection.

Study design

Adult interferon α/β receptor-deficient IFNAR-/- mice[10] were injected *i. p.* with 500 µl of serial dilutions of culture-grown BTV-6 or BTV-8.

Five BTV-naïve German Blackhead mutton sheep were inoculated with 5×10^5 $TCID_{50}$ of a 2008 German isolate of BTV-8[11].

In a second experiment, 5 BTV-naïve sheep, 5 sheep that had been vaccinated with an inactivated BTV-8 vaccine 7 weeks previously, and 5 sheep that had been experimentally infected with BTV-8 5 weeks before were challenged with 5×10^5 $TCID_{50}$ of German BTV-6[14].

Differences between groups were evaluated with one-sided Wilcoxon rank-sum tests.

Results

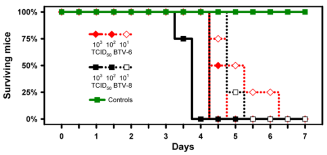

Figure 2: Survival times of mice after BTV infection. As little as five $TCID_{50}$ of BTV-6 or BTV-8 killed all mice.

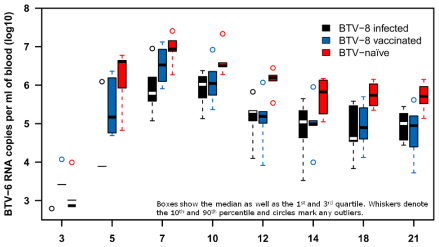

Figure 3: Results of a BTV-6 specific real-time RT-PCR[12] for naïve and BTV-8 exposed sheep. BTV-6 replication was reduced in previously exposed animals.

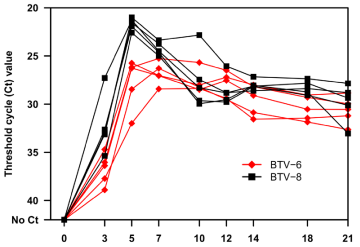

Figure 4: Real-time RT-PCR[13] results for BTV-naïve sheep infected with BTV-6 or BTV-8.

Conclusions

In BTV-naïve sheep, BTV-6 replicates at a lower level than BTV-8. Their equivalent virulence in IFNAR-/- mice, however, suggests that the attenuation of BTV-6 is related to the JAK-STAT pathway.

Exposure to BTV-8 interferes with subsequent BTV-6 infection. In sheep that had been previously infected with BTV-8, BTV-6 RNA levels were significantly reduced throughout the experiment. In BTV-8 vaccinated sheep, the amount of BTV-6 RNA in circulation was significantly lower than in naïve animals after day 10 post infection. Generally, bluetongue viremia is prolonged because virus particles passively associate with blood cells[14].

The restrictive influence of previous BTV-8 exposure and the reduced replication efficiency of BTV-6 in the naïve host offer a possible explanation for its failure to spread in the wake of the BTV-8 epizootic in Europe.

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5 Discussion

5.1 Bluetongue vaccination

5.1.1 Short- and long-term efficacy of inactivated vaccines

When the inactivated BTV-8 vaccines were first delivered in 2008, no peer-reviewed data on their safety and efficacy were available. The study presented here was the first independent assessment of the efficacy of the vaccines used in Germany and elsewhere (Eschbaumer *et al.*, 2009a). Complementing these results, a study demonstrating the safety and efficacy of their company's vaccine was later published by researchers from Merial (Hamers *et al.*, 2009a).
Four manufacturers had offered inactivated vaccines against BTV-8, but only three were available in Germany in 2008. In a recent publication, the fourth product (Bovilis® BTV8, Intervet International b.v., Boxmeer, The Netherlands) was shown to protect sheep against virulent challenge ten months after vaccination (Oura *et al.*, 2009), the first published study of long-term efficacy of any inactivated vaccine against BTV-8. The 12-month study presented here is the first to provide data on the long-term efficacy in cattle. It further confirmed the findings of Oura *et al.* for the three other vaccines in sheep. Two of the vaccines (by Merial and Fort Dodge) have since been issued marketing authorizations valid throughout the EU (EMEA, 2010a; b; c).
The excellent experimental efficacy is overwhelmingly corroborated by field data. After the safety of the vaccines had been demonstrated (Gethmann *et al.*, 2009), they were temporarily exempted from German licensing requirements (BlZSchutzImpfuaÄndV, 2008). Vaccinations began in mid-May, and over 80% coverage had been achieved by the end of 2008 (Gethmann *et al.*, 2010). This is generally regarded as the critical threshold for a BTV vaccination campaign (Giovannini *et al.*, 2004). From 22,700 cases in the previous year, the number of new BTV cases notified in Germany between May 2008 and April 2009 dropped to 3,200 and the virus did not spread further east (see maps in Conraths *et al.*, 2009). Apart from vaccination, natural immunity after infection contributed to the reduction of new cases in areas that had been severely affected in 2007 (Conraths *et al.*, 2009).
Vaccinations in Germany continued in 2009, and between May and October 2009, less than 10 new cases had been reported (Gethmann *et al.*, 2010), while there is no compelling evidence that any of those actually was a productive BTV infection[1]. See figure 6 for a timeline of BTV-8 cases in Germany.

[1] Bernd Hoffmann, German NRL BT, personal communication.

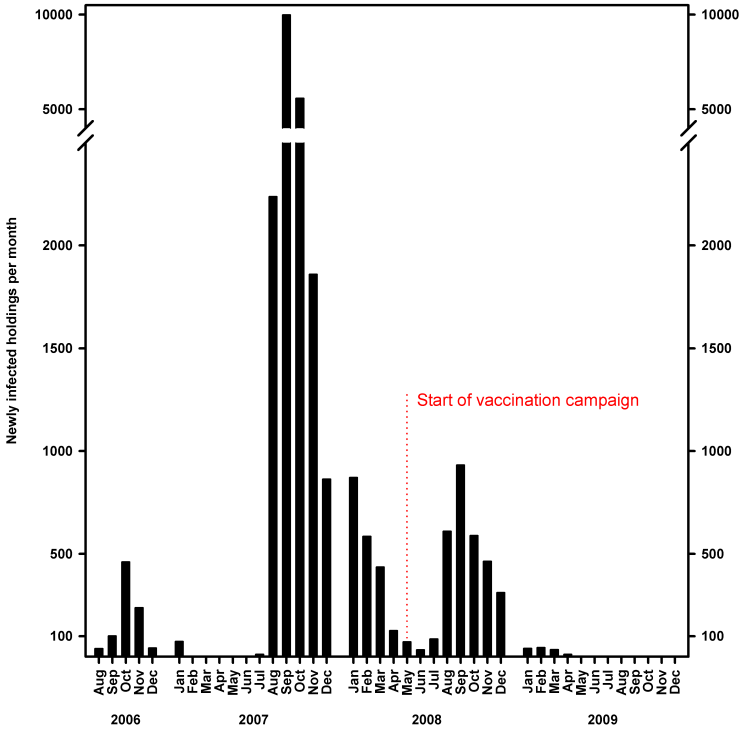

Figure 6: BTV-8 cases (infected animal holdings) in Germany from 2006 to 2009 (Anonymous, 2007; 2008; 2009a; b).

Conversely, in France, where vaccination was not compulsory and coverage was low, the number of new cases increased from 21,000 in 2007/2008 to 29,000 in 2008/2009. Gethmann *et al.* (2010) give an exhaustive overview of case numbers and vaccination coverage in Europe. Overall, the BTV-8 vaccination campaign is a remarkable success story. This success, however, is now put in jeopardy by the hasty decision to abandon compulsory vaccination (Mettenleiter, 2009).

5.1.2 Vaccination and impact on diagnostic capability

The commonly used commercial ELISA systems for BTV antibody detection are based on VP7 (Batten *et al.*, 2008a). This is a structural protein, and both BTV infection and vaccination with inactivated whole virus vaccines will create a VP7 antibody response. Therefore, these systems do not allow the differentiation of infected from vaccinated animals (DIVA) for the currently available BTV-8 vaccines, and meaningful serological surveillance is rendered impossible by the vaccination campaigns (Mertens *et al.*, 2009a).
On the other hand, since inactivated vaccines do not cause virus replication, there is no perceived risk of them interfering with molecular diagnostics, but this has not been studied in depth. In one published study, the community reference laboratory found no BTV genome in 33 sheep at one day or one week after vaccination (Oura *et al.*, 2009). This was confirmed by the experiment presented here (Eschbaumer *et al.*, 2010). Another study in sheep, however, had detected BTV RNA in recently vaccinated animals (Steinrigl *et al.*, 2009).
In any case, the practical relevance of this matter is limited. Even in the latter study, vaccine-derived RNA was only frequently detectable within the first two weeks after vaccination. In this time frame, however, vaccinated animals will not routinely be examined with real-time RT-PCR. For animal movement in the European Union, the relevant guidelines are given in regulation 289/2008 (European Commission, 2008). When no testing is done, vaccinated animals are not be moved until 60 days after vaccination. If alternatively they are to be tested for virus in order to be moved earlier, this can first be done 14 days after the onset of immunity as laid out in the vaccine specifications (15 to 25 days, depending on the manufacturer). The OIE Terrestrial Animal Health Code, on the other hand, does not grant any exemption from the 60-day rule at all (OIE, 2009a).

5.1.3 Challenge experiments

The breeding of *Culicoides* midges in captivity is very demanding, and only a small number of colonies have been established worldwide (Veronesi *et al.*, 2009). Accordingly, most experimental infections with BTV are carried out by needle inoculation (reviewed by Darpel *et al.*, 2009a) of blood from viremic ruminants or culture-grown virus.
In nature, BTV is maintained by an endless series of alternating cycles of replication in *Culicoides* midges and various mammalian ruminant species

(Mellor, 1990) and the virus has evolved to maximum efficiency in this context. Other means of propagation, including repeated passages in ruminants alone, change the selective pressure on the virus quasispecies. Point mutations introduced during viral replication lead to initially subtle differences in replication efficiency of virus progeny, and repeated passages will favor strains that replicate efficiently in the heterologous culture system (Dungu *et al.*, 2004). While the phenotypic changes induced by frequent passaging in heterologous culture systems (Kirkland and Hawkes, 2004) are long known and have been used for the production of attenuated vaccines for decades (Dungu *et al.*, 2004), they are only now being investigated on a molecular basis (Caporale *et al.*, 2009). Even though dramatic changes in fitness resulting from single point mutations have been described (Le Blois and Roy, 1993; Tsetsarkin *et al.*, 2007), virus attenuation often is a gradual process and the number of passages required for a noticeable reduction in virulence is variable (Kirkland and Hawkes, 2004).

Presently, given the dubious history of the European BTV-8 field strain, it is doubtful whether a small number of culture passages will result in dramatic changes to its phenotype. A case can be made that for this strain the benefits of virus maintenance in culture – easier preparation, standardization, handling and storage of inocula – outweigh the risk of further misadaptation of the virus. Accordingly, the study presented here found no difference in virulence between a native BTV isolate passaged only in ruminants and culture-grown virus at a low passage level, making the latter a viable choice for challenge experiments.

The reduction of suffering in control animals in a challenge experiment is a welfare issue. The OIE requirement of clinical disease in control animals as proof of successful challenge infection is rendered obsolete by advances in molecular detection techniques. Conversely, the replacement of ruminants in BTV animal experiments by rodents only offers economical advantages. But these can be considerable, in terms of both purchase and maintenance. It has recently been shown that interferon-α/β receptor-deficient mice are fully susceptible to BTV infection (Calvo-Pinilla *et al.*, 2009a; independently confirmed in the study presented here), but can be protected by vaccination (Calvo-Pinilla *et al.*, 2009b). This makes them a very promising tool for vaccine research and evaluation, and possibly even pathogenesis studies. Nevertheless, they are not the natural host, and conclusions based on observations in the mouse model will eventually require confirmation in sheep or cattle.

5.2 Bluetongue diagnosis and epizootiology

5.2.1 Highly sensitive detection and serotyping by real-time RT-PCR

In the past, the identification of BTV in a field sample was a labor-intensive and time-consuming process. Goldsmit and Barzilai (1968) put the time required at five to nine weeks. Some forty years later, twenty years after the first adaptation of the PCR method to BTV detection (Dangler *et al.*, 1990), real-time RT-PCR can detect and type BTV in any sample in a matter of hours. Its combination of speed, sensitivity, and specificity (Bustin *et al.*, 2009) has transformed BTV diagnostics. Molecular methods (real-time RT-PCR as well as RT-PCR and sequencing) are now the standard for BTV antigen detection and characterization. Traditional methods retain an important role as confirmatory tests, however. Due to the very high sensitivity of the real-time RT-PCR, the clinical and epidemiological significance of weakly positive results must be carefully evaluated, especially if no virus can be isolated (Eschbaumer *et al.*, 2009a).

The assays presented here were the first published assays to specifically detect BTV-1, 6 and 8 (Hoffmann *et al.*, 2009c). An earlier assay developed at the Friedrich-Loeffler-Institut (first published in Conraths *et al.*, 2009, but actually developed in 2006) was highly sensitive but not strictly specific for BTV-8. Previously, only one type-specific BTV real-time RT-PCR assay had been made public, detecting and differentiating BTV-2 field and vaccine strains (Orrù *et al.*, 2004). The new assays are used in routine diagnostics in the national reference laboratory and regional laboratories and have proven to be very reliable.

Meanwhile, another set of assays specific for BTV serotypes 1, 6, 8 and 11 has been presented (Vandenbussche *et al.*, 2009), and a commercial kit that can differentiate between BTV-1, 2, 6, 8, 9, 11 and 16 is now available (Anonymous, 2009i).

5.2.2 BTV-6 and -11 in Europe

After the initial detection of BTV-6 and BTV-11, extensive field surveys found only very low prevalences for both viruses (De Clercq *et al.*, 2009; Eschbaumer *et al.*, 2009b). Neither virus reappeared in 2009, confirming the passive stance taken by the veterinary authorities (SCFCAH, 2008).
The very high level of genetic identity between their segments 2 and the respective modified-live vaccine strains had been noted very early on (ProMED-mail, 2008; 2009). With the detection of segment 10 from the BTV-2 vaccine strain in the European isolates of BTV-6 (Maan *et al.*, 2010), evidence for vaccine strains from all three bottles of the Onderstepoort Biological Products vaccine package has now been found in Northern Europe. It is possible that the reassortment occurred during co-infection by BTV-2 and BTV-6 vaccine strains, which would corroborate the theory that the illegal use of live vaccines lies at the heart of the recent emergence of two new BTV serotypes in Europe. The manufacturer of the vaccine package states that no vaccine has recently been delivered to Europe. It is freely available in agricultural stores in South Africa, however, and the importation of small amounts in tourist travel cannot be ruled out (Anonymous, 2009g).
The level of variation observed in segment 10 (from BTV-2) in Dutch BTV-6, on the other hand, suggests that the exchange had happened several cycles of replication before the virus arrived in Europe (Maan *et al.*, 2010). Unfortunately, no isolate of Belgian BTV-11 could be obtained. Therefore, only its segment 2 was partially sequenced, and no data on its other segments are available (De Clercq *et al.*, 2009).
In any case, a vaccine origin alone cannot fully explain the failure of BTV-6 to spread after its introduction into the Netherlands and Germany. Other MLV strains had been shown to be pathogenic for European breeds of sheep (Veronesi *et al.*, 2005; Veronesi *et al.*, 2010), and have been widely spread by vectors (Ferrari *et al.*, 2005; Listes *et al.*, 2009). The influence of a previous exposure to BTV-8 observed in the study presented here and the reduced replication efficiency of BTV-6 in naïve animals offer a possible explanation for its failure to spread in the wake of the BTV-8 epizootic. A similar hypothesis was put forward for BTV-11 (De Clercq *et al.*, 2009).
While the recent emergence of BTV-6 and BTV-11 in the same geographical region might be attributable to the illegal use of live vaccines, there is contradictory molecular evidence. More data on the time and place of the introduction of BTV-8 have recently become available (Saegerman *et al.*, 2010b), but its origin and particularly the agent and method of introduction

remain mysterious as ever. These, however, are the key issues with regard to future introduction events of BTV, other arboviruses, or non-viral vector-borne diseases. A particularly alarming scenario is the introduction of African horse sickness. It is caused by a closely related orbivirus that is regularly spread by the same vectors (Mellor and Hamblin, 2004), so it could conceivably be introduced in the same way as BTV. Its impact on the naïve horse population in Europe would be tremendous, with expected mortalities of up to 95% (recently reviewed by Zimmerli *et al.*, 2010).
Vector-borne pathogens pose exceptional challenges to biosafety and disease control. Further research into the means and ways of introduction of foreign animal diseases into the European Union is absolutely vital, as is the establishment of suitable vaccine reserves.

5.3 Conclusions and outlook

Vaccination with inactivated vaccines proved to be a highly effective tool in BTV-8 control. Data collected in the initial safety study and during the German vaccination campaign showed very good safety for all vaccines. This was confirmed by data from several other EU member countries (EMEA, 2009) and by a field study in Switzerland (Bruckner *et al.*, 2009). In 2008, the incidence of adverse reactions notified to the competent authority in Germany (Paul-Ehrlich-Institut) was only 0.003% (Hoffmann and Cußler, 2009). Individual farmers, however, reported massive damage to their stock. Studies in Switzerland and Germany followed up on these reports, and found no causal link to BTV vaccination. To the contrary, it was felt that farmers often used BTV vaccination as a simple explanation for complex management problems (Probst *et al.*, 2010). Interestingly, some farmers who had publicly decried vaccination subsequently declined the offered on-site investigations (Tschuor *et al.*, 2009).
Public perception, however, was different. A campaign of disinformation and fearmongering mounted by a vocal minority (see Englhart, 2009 for an example) received wide media coverage. Despite its excellent success in 2008 and 2009, the compulsory vaccination campaign in Germany was discontinued following a majority vote by the states, contrary to unanimous recommendations by the Federal Ministry, veterinary associations, farming bodies and the Federal Research Institute for Animal Health (Anonymous, 2009j). The original goal of the campaign, the eradication of BTV-8, was summarily abandoned.
There is a high presumed risk for the reoccurrence and spread of BTV-8 in Germany in 2011 if there is a large decrease in vaccine coverage in 2010 (Mettenleiter, 2009). Licensed vaccines are now available in the market,

however, and the future development will depend on the farmers' willingness to vaccinate their stock and the duration of immunity afforded by previous infections and vaccinations, not only in Germany but in all of Europe (Gethmann *et al.*, 2010). As an incentive, several federal states will continue to cover the costs for BTV vaccines in 2010 (Anonymous, 2009c; f; 2010a; b). Another important factor in the recrudescence is the possible virus reservoir in wild ruminants, but the available data currently bear out no definite conclusion in this regard (Mettenleiter, 2009).

In any case, this pertains only to BTV-8. If a virulent strain of a new serotype of BTV is introduced to Germany, vaccination will again become the only option. Recent events have shown that introductions can happen at any time with no warning at all, and further research on possible routes and ways of introduction of vector-borne diseases is critical.

The recent development of reverse genetic systems for BTV (Boyce *et al.*, 2008; Attoui *et al.*, 2009) has opened up tremendous possibilities for research into BTV replication (Matsuo and Roy, 2009), pathogenesis (Caporale *et al.*, 2009) and novel vaccines (Roy *et al.*, 2009). The BTV-8 epizootic in Europe has sparked interest and brought about funding for BTV research like no outbreak of this disease before. Concerted efforts of scientists all over the world have provided new insights that will directly benefit global animal health.

6 Summary

Compulsory vaccination with inactivated vaccines against BTV-8 had been introduced in Germany in mid-2008. The used vaccines were shown to be very efficacious, and immunity was found to persist for at least one year, even in sheep that had been given only one dose of vaccine. It is considered unlikely that vaccination with inactivated BTV-8 vaccines interferes with routine diagnostics by real-time RT-PCR. In preparation of future challenge experiments, culture-grown BTV-8 and virus passaged only in ruminants were compared. There was no pronounced difference in the induction and progression of viremia in sheep. Both inocula were also shown to fatally infect interferon-α/β receptor-deficient mice, confirming the utility value of the knock-out mouse model for BTV research.
After the introduction of BTV-6 to northwestern Germany in 2008, an extensive field survey was carried out. The prevalence of BTV-6 in Lower Saxony was found to be very low, and it did not spread any further in 2009. The virus was shown to be derived from a modified-live vaccine strain. Based on experimental findings, its failure to spread is probably attributable to the previous BTV-8 epizootic in the region. This and other studies were only made possible by the development and validation of serotype-specific real-time RT-PCR assays for the highly sensitive detection of BTV-1, -6 and -8. They have been transferred to regional veterinary laboratories and can be used in routine diagnostics.

This work reproduces major recent contributions to BTV research. Experimental findings fully supported the decision to use inactivated vaccines for the EU campaign. Studies of BTV virulence and challenge models provided vital data for future experiments. The implications of the introduction of BTV-6 into Germany were evaluated in detail, and the newly developed real-time RT-PCR assays considerably extended the possibilities of BTV diagnostics in the European context.

7 Zusammenfassung

Mitte 2008 wurde die verpflichtende Impfung gegen BTV-8 in Deutschland eingeführt. Die hohe Wirksamkeit der verwendeten Impfstoffe konnte gezeigt werden. Nach Anwendung der Vakzine hielt der Impfschutz mindestens ein Jahr an, sogar bei Schafen die nur eine Impfdosis erhalten hatten. Es erscheint unwahrscheinlich, dass sich eine Impfung mit inaktivierten BTV-8-Impfstoffen störend auf die Routinediagnostik mittels Real-time RT-PCR auswirkt. Zur Vorbereitung weiterer Impfstoffversuche wurde BTV-8 aus Zellkultur mit einem nur in Wiederkäuern passagierten Isolat verglichen. Bei Schafen konnte kein Unterschied im Infektionsverlauf festgestellt werden. Außerdem konnte mit beiden Isolaten eine tödliche Infektion in Mäusen ohne Interferon-α/β-Rezeptor hervorgerufen werden, was den Nutzwert des Knock-out-Mausmodells für die BTV-Forschung bestätigt.
Nach der Einschleppung von BTV-6 nach Niedersachsen im Jahr 2008 wurde eine umfassende Feldstudie durchgeführt. Dabei wurde nur eine sehr niedrige Prävalenz festgestellt und das Virus breitete sich 2009 nicht weiter aus. Die Ergebnisse weitergehender Untersuchungen lassen vermuten, dass die Verbreitung des als Impfstamm identifizierten Virus durch die hohe Durchseuchung mit BTV-8 in der Region verhindert wurde. Diese und andere Studien waren nur möglich, nachdem serotyp-spezifische Real-time RT-PCR-Verfahren für die hochsensitive Erkennung von BTV-1, -6 und -8 entwickelt und erfolgreich getestet worden waren. Die Verfahren wurden an die regionalen Untersuchungsämter gegeben und können in der Routinediagnostik eingesetzt werden.

Diese Arbeit fasst wichtige aktuelle Beiträge zur BTV-Forschung zusammen. Die Impfstoffversuche bestätigten umfassend die Entscheidung, inaktivierte Impfstoffe für die EU-Kampagne einzusetzen. Die Untersuchungen zu Challengemodellen und Virulenz von BTV lieferten wertvolle Daten für zukünftige Versuche. Die Auswirkungen der Einschleppung von BTV-6 nach Deutschland wurden ausführlich untersucht, und die neu entwickelten Real-time RT-PCR-Verfahren erweiterten die diagnostischen Möglichkeiten für BTV in Europa erheblich.

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9 Abbreviations

aa	Amino acids
AHSV	African Horse Sickness virus
BHK	Baby hamster kidney
BMELV	Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz
BT	Bluetongue disease
BTV	Bluetongue virus
cDNA	Complementary desoxyribonucleic acid
CLP	Core-like particle
CTL	Cytotoxic T-lymphocyte
DIVA	Differentiating infected from vaccinated animals
DNA	Deoxyribonucleic acid
EC	Endothelial cells
EHDV	Epizootic Hemorrhagic Disease virus
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
EU	European Union
(m)RNA	(Messenger) ribonucleic acid
MLV	Modified-live virus
NS	Non-structural protein
OIE	World Organisation for Animal Health
PBMC	Peripheral blood mononuclear cells
RBC	Red blood cells
RT-PCR	Reverse transcription polymerase chain reaction
SA	South African
SCAHAW	Scientific Committee on Animal Health and Animal Welfare
SCFCAH	Standing Committee on the Food Chain and Animal Health
SNT	Serum neutralization test
spp.	*Species pluralis*, several species
TOV	Toggenburg orbivirus
US, U.S.	United States
VLP	Virus-like particle
VP	Virion protein

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