Accepted Manuscript

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

Authors: Michael Eschbaumer, Bernd Hoffmann, Andreas Moss, Giovanni Savini, Alessandra Leone, Patricia König, Johanna Zemke, Franz Conraths, Martin Beer

PII: S0378-1135(09)00589-6
DOI: doi:10.1016/j.vetmic.2009.11.040
Reference: VETMIC 4695

To appear in: *VETMIC*

Received date: 13-9-2009
Revised date: 16-11-2009
Accepted date: 26-11-2009

Please cite this article as: Eschbaumer, M., Hoffmann, B., Moss, A., Savini, G., Leone, A., König, P., Zemke, J., Conraths, F., Beer, M., Emergence of bluetongue virus serotype 6 in Europe–German field data and experimental infection of cattle, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.11.040

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Michael Eschbaumer¹, Bernd Hoffmann¹, Andreas Moss², Giovanni Savini³,
Alessandra Leone³, Patricia König¹, Johanna Zemke¹, Franz Conraths⁴, and Martin
Beer^{1,*}

¹Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493
Greifswald-Insel Riems, Germany

²LAVES - Veterinary Institute Oldenburg, Philosophenweg 38,
26121 Oldenburg, Germany

³Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via
Campo Boario, 64100 Teramo, Italy

⁴Institute of Epidemiology, Friedrich-Loeffler-Institut, Seestraße 55,
16868 Wusterhausen, Germany

*Corresponding author:

Telephone: +49 38351 7200, Fax: +49 38351 7226, E-mail: martin.beer@fli.bund.de

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.

Keywords: bluetongue virus / btv-6 / germany / bovine / field data

1. Introduction

1.1. 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.

1.2. 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.

2. Materials and Methods

2.1. Field survey

Initially, infection with BTV-6 was detected in four cattle in three holdings in the district Graftschaft 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).

2.2. Animal experiment

2.2.1. 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® AISap 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.

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.

2.2.2. 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).

2.2.3. 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.

3. Results

3.1. 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.

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.

3.2. 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). 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.

4. 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 defined challenge

doses of BTV-1 and -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.

5. 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.

Acknowledgements

The authors thank the technical staff at the Friedrich-Loeffler-Institut and the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise for their outstanding work, and Jürgen Teuffert and Carolina Probst (Institute of Epidemiology of the Friedrich-Loeffler-Institut, Wusterhausen) for providing epidemiological field data. Mario Ziller's help with the statistical analysis of the data is also gratefully acknowledged. This work was supported by EPIZONE, the EU Network of Excellence for Epizootic Disease Diagnosis and Control (FOOD-CT-2006-016236).

References

- Clavijo, A., Heckert, R.A., Dulac, G.C., Afshar, A., 1999. Isolation and identification of bluetongue virus. *J. Virol. Methods* 87, 13-23.
- Dal Pozzo, F., Saegerman, C., Thiry, E., 2009. Bovine infection with bluetongue virus with special emphasis on European serotype 8. *Vet. J.* doi:10.1016/j.tvjl.2009.05.004.
- Darpel, K.E., Batten, C.A., Veronesi, E., Shaw, A.E., Anthony, S., Bachanek-Bankowska, K., Kgosana, L., bin-Tarif, A., Carpenter, S., Müller-Doblies, U.U., Takamatsu, H.H., Mellor, P.S., Mertens, P.P., Oura, C.A., 2007. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. *Vet. Rec.* 161, 253-261.
- Dungu, B., Gerdes, T., Smit, T., 2004. The use of vaccination in the control of bluetongue in southern Africa. *Vet. Ital.* 40, 616-622.
- Eschbaumer, M., Hoffmann, B., König, P., Teifke, J.P., Gethmann, J.M., Conraths, F.J., Probst, C., Mettenleiter, T.C., Beer, M., 2009. Efficacy of inactivated vaccines against bluetongue virus serotype 8 in sheep. *Vaccine* 27, 4169-4175.
- Gard, G.P., Kirkland, P.D., 1993. Bluetongue virology and serology. In: Corner, L.A., Bagust, T.J. (Eds.), *Australian Standard Diagnostic Techniques for Animal Diseases*, CSIRO Information Services, Melbourne, Australia.

- Hateley, G., 2009. Bluetongue in northern Europe: the story so far. In Practice 31, 202-209.
- Hoffmann, B., Beer, M., Reid, S.M., Mertens, P., Oura, C.A., van Rijn, P.A., Slomka, M.J., Banks, J., Brown, I.H., Alexander, D.J., King, D.P., 2009a. A review of RT-PCR technologies used in veterinary virology and disease control: Sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Vet. Microbiol. 139, 1-23.
- Hoffmann, B., Eschbaumer, M., Beer, M., 2009b. Real-Time Quantitative Reverse Transcription-PCR Assays Specifically Detecting Bluetongue Virus Serotypes 1, 6, and 8. J. Clin. Microbiol. 47, 2992-2994.
- Hofmann, M.A., Renzullo, S., Mader, M., Chaignat, V., Worwa, G., Thuer, B., 2008. Genetic Characterization of Toggenburg Orbivirus, a New Bluetongue Virus, from Goats, Switzerland. Emerg. Infect. Dis. 14, 1855-1861.
- International Society for Infectious Diseases (ISID), Program for Monitoring Emerging Diseases (ProMED-mail), 2008a. Bluetongue - Europe (61): Netherlands, new strain susp, RFI. Archive number 20081018.3301. <http://www.promedmail.org> [posted 18 October 2008]

- 393 ISID ProMED-mail, 2008b. Bluetongue - Europe (64): Netherlands, BTV-6. Archive
 394 number 20081024.3364. <http://www.promedmail.org> [posted 24 October 2008]
 395
- 396 ISID ProMED-mail, 2008c. Bluetongue - Europe (66): Netherlands, BTV-6 vaccine
 397 strain susp. Archive number 20081028.3410. <http://www.promedmail.org> [posted 28
 398 October 2008]
 399
- 400 ISID ProMED-mail, 2008d. Bluetongue - Europe (69): Netherlands, BTV-6 vaccine
 401 strain update. Archive number 20081031.3431. <http://www.promedmail.org> [posted 31
 402 October 2008]
 403
- 404 ISID ProMED-mail, 2008e. Bluetongue - Europe (75): Netherlands, BTV-6. Archive
 405 number 20081113.3580. <http://www.promedmail.org> [posted 13 November 2008]
 406
- 407 ISID ProMED-mail, 2009. Bluetongue - Europe (05): Belgium, BTV-11. Archive
 408 number 20090218.0684. <http://www.promedmail.org> [posted 18 February 2009]
 409
- 410 Kroschewski, K., Kramer, M., Micklich, A., Staubach, C., Carmanns, R., Conraths,
 411 F.J., 2006. Animal disease outbreak control: the use of crisis management tools. Rev.
 412 Sci. Tech. Off. Int. Epiz. 25, 211-221.
 413
- 414 Listeš, E., Monaco, F., Labrović, A., Paladini, C., Leone, A., Di Gialleonardo, L.,
 415 Cammá, C., Savini, G., 2009. First evidence of bluetongue virus serotype 16 in Croatia.
 416 Vet. Microbiol. 138, 92-97.

417
 418 Maan, S., Maan, N.S., Ross-Smith, N., Batten, C.A., Shaw, A.E., Anthony, S.J.,
 419 Samuel, A.R., Darpel, K.E., Veronesi, E., Oura, C.A.L., Singh, K.P., Nomikou, K.,
 420 Potgieter, A.C., Attoui, H., van Rooij, E., van Rijn, P., De Clercq, K., Vandenbussche,
 421 F., Zientara, S., Bréard, E., Sailleau, C., Beer, M., Hoffmann, B., Mellor, P.S., Mertens,
 422 P.P.C., 2008. Sequence analysis of bluetongue virus serotype 8 from the Netherlands
 423 2006 and comparison to other European strains. *Virology* 377, 308–318.
 424
 425 MacLachlan, N.J., Drew, C.P., Darpel, K.E., Worwa, G., 2009. The Pathology and
 426 Pathogenesis of Bluetongue. *J. Comp. Pathol.* 141, 1-16.
 427
 428 MinLNV, Dutch Ministry of Agriculture, Nature and Food Quality, 2009a. BTV 6 in
 429 the Netherlands. [http://ec.europa.eu/food/committees/regulatory/scfcach/animal_health/](http://ec.europa.eu/food/committees/regulatory/scfcach/animal_health/presentations/bt_34032009_nl.pdf)
 430 [presentations/bt_34032009_nl.pdf](http://ec.europa.eu/food/committees/regulatory/scfcach/animal_health/presentations/bt_34032009_nl.pdf) [accessed 1 September 2009]
 431
 432 MinLNV, 2009b. Epidemiological report BTV 6 in the Netherlands.
 433 [http://ec.europa.eu/food/animal/diseases/controlmeasures/docs/epidemiological_report_](http://ec.europa.eu/food/animal/diseases/controlmeasures/docs/epidemiological_report_en.pdf)
 434 [en.pdf](http://ec.europa.eu/food/animal/diseases/controlmeasures/docs/epidemiological_report_en.pdf) [accessed 1 September 2009]
 435
 436 Mintiens, K., Méroc, E., Mellor, P.S., Staubach, C., Gerbier, G., Elbers, A.R.W.,
 437 Hendrickx, G., De Clercq, K., 2008. Possible routes of introduction of bluetongue virus
 438 serotype 8 into the epicentre of the 2006 epidemic in north-western Europe. *Prev. Vet.*
 439 *Med.* 87, 131-144.
 440

- Monaco, F., De Luca, N., Spina, P., Morelli, D., Liberatore, I., Citarella, R., Conte, A., Savini, G., 2004. Virological and serological response of cattle following field vaccination with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9. *Vet. Ital.* 40, 657-660.
- Saegerman, C., Berkvens, D., Mellor, P.S., 2008. Bluetongue Epidemiology in the European Union. *Emerg. Infect. Dis.* 14, 539-544.
- Saegerman, C., Pastoret, P.P., 2009. Bluetongue in northern Europe: appearance of new serotypes within an enzootic ground. *Bull. Off. Int. Epiz.* 1, 35-39.
- Savini, G., MacLachlan, N.J., Sánchez-Vizcaíno, J.M., Zientara, S., 2008. Vaccines against bluetongue in Europe. *Comp. Immunol. Microbiol. Infect. Dis.* 31, 101-120.
- Schwartz-Cornil, I., Mertens, P.P.C., Contreras, V., Hemati, B., Pascale, F., Bréard, E., Mellor, P.S., MacLachlan, N.J., Zientara, S., 2008. Bluetongue virus: virology, pathogenesis and immunity. *Vet. Res.* 39, 46.
- SCoFAH, Standing Committee on the Food Chain and Animal Health of the European Commission, 2009. Statement on BTV6 and BTV11. http://ec.europa.eu/food/committees/regulatory/scfcah/animal_health/presentations/bt_statement_btv6-11.pdf [accessed 1 September 2009]

- Singer, R.S., MacLachlan, N.J., Carpenter, T.E., 2001. Maximal predicted duration of viremia in bluetongue virus-infected cattle. *J. Vet. Diag. Invest.* 13, 43-49.
- Toussaint, J.F., Sailleau, C., Bréard, E., Zientara, S., De Clercq, K., 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments, *J. Virol. Methods* 140, 115-123.
- Van Rijn, P., 2009. Animal Trials Blue Tongue Serotype 6 in the Netherlands. http://ec.europa.eu/food/committees/regulatory/scfcah/animal_health/presentations/bt_34022009_nl.pdf [accessed 1 September 2009]
- Veronesi, E., Hamblin, C., Mellor, P.S., 2005. Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). *Vaccine* 23, 5509-5516.
- Verwoerd, D.W., Erasmus, B.J., 2004. Bluetongue. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), *Infectious Diseases of Livestock* (2nd ed.), Oxford University Press Southern Africa, Cape Town, South Africa.

Table captions

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.

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.

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.

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).

506 **Figure captions**

507

508 Figure 1: Cattle herds with BTV-6 infections in Germany

509

510 Dots (blue) mark the locations of affected holdings. The thick line (red) is the Dutch-

511 German border.

Table I

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 II

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 III

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 IV

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

Figure 1