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Experimental infection of white-tailed deer (*Odocoileus virginianus*) with Northern European bluetongue virus serotype 8☆

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ABSTRACT

Bluetongue (BT) is an insect-transmitted, economically important disease of domestic and wild ruminants. Although only five of the 26 reported bluetongue virus (BTV) serotypes are considered endemic to the USA, 10 exotic serotypes have been isolated primarily in the southeastern region of the country since 1999. For an exotic BTV serotype to become endemic there must be susceptible animal species and competent vectors. In the USA, sheep and white-tailed deer (WTD) are the primary sentinel livestock and wildlife species, respectively. In 2006, BTV-8 was introduced into Northern Europe and subsequently overwintered, causing unprecedented livestock disease and mortality during the 2006–2007 vector seasons. To assess the risk of the European strain of BTV-8 to North American WTD, and understand the role they could play after a similar introduction, eight bluetongue-seronegative WTD were inoculated with BTV-8. Body temperatures and clinical signs were recorded daily. Blood samples were analyzed for BTV RNA with quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR), serum analyzed for BTV antibodies by cELISA, and tissues taken for histopathology and qRT-PCR. All eight deer became infected and developed moderate to severe clinical disease from days 8 to 15. Peak viremia was from day 7 to 10 with detectable titers through the end of the study (28 days) in most deer. Serum antibody was detected by day 6, peaked by day 10 and continued through day 28. We conclude that North American WTD are highly susceptible to BTV-8 and would act as clinical disease sentinels and amplifying hosts during an outbreak.

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1. Introduction

Bluetongue virus (BTV) is an orbivirus that is transmitted by biting midges (*Culicoides* spp.) and causes transboundary, internationally reportable disease with high morbidity and mortality in susceptible animal populations. Serious economic impacts result directly from decreased animal production, as well as indirectly from resulting

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regulatory repercussions. Bluetongue (BT) poses a constant disease threat to the livelihood of livestock owners.

While *Culicoides* midges feed on numerous domestic and wild ruminants (Tabachnick, 2004), BT in the USA is largely a disease of sheep, cattle, deer (*Odocoileus* spp.) and pronghorn antelope (*Antilocapra americana*). In domestic animals, clinical disease is primarily seen in sheep and includes fever, nasal lesions and discharge, excessive salivation, anorexia, dehydration, depression, coronitis, facial edema, conjunctivitis, cyanotic (blue) tongue, and secondary pneumonia with up to 50% mortality (Parsonson, 1990). In wildlife, overt clinical disease is primarily seen in white-tailed deer (WTD; *O. virginianus*) and pronghorn antelope (Hoff and Trainer, 1978; Kocan et al., 1987; Thorne et al., 1988; Stallknecht and Howerth, 2004). These wildlife species play important roles in the epidemiology of BT outbreaks as sentinels, amplifying hosts, and viral reservoirs in close proximity to sheep and cattle grazing and watering areas. Similar to cattle, other wild ruminant species may have histopathological indication of disease upon necropsy, but rarely show overt clinical disease and are considered potential sub-clinical viral reservoirs (Hoff and Trainer, 1972, 1978).

There are 26 reported serotypes of BTV, five of which are considered endemic to the USA (BTV-2, 10, 11, 13, and 17) (Collisson and Barber, 1985). Morbidity and mortality rates observed during outbreaks from different serotypes are highly variable and with little to no serotype cross-protection reported to date using current vaccine technologies, vaccination must be serotype-specific (Noad and Roy, 2009). The global distribution of BTV serotypes has changed drastically in the past 15 years, possibly as a result of climate change (Purse et al., 2005, 2008; Gale et al., 2010). The rapid spread of BTV-8 throughout Northern Europe in 2006–2007 is particularly alarming as the risk to livestock and wildlife was thought to be minimal or non-existent. This was the largest single serotype outbreak ever recorded with devastating disease and mortality in sheep and cattle. In Germany alone, the 2007 cases in cattle, sheep and goats were 26,772, 32,116 and 209 with case-fatality rates of 13%, 42% and 26%, respectively (Conraths et al., 2009). It is estimated that the losses to the agriculture industry was over 200 million Euros (Velthuis et al., 2010).

The United States is at risk for similar incursions. Since 1999, 10 exotic BTV serotypes have been detected in the southeastern region of the country: BTV-1, 3, 5, 6, 9, 12, 14, 19, 22, and 24 (Ostlund, 2010). Introduction of these new serotypes may have resulted from the importation of infected livestock (Hoar et al., 2004), or the arrival of infected midges *via* wind currents (Sellers, 1980; Sellers and Maarouf, 1989), shipping containers, or cargo holds of ships as has been reported for some mosquito introductions (Linthicum et al., 2003; Furumizo et al., 2005).

In order to assess whether an exotic BTV introduction into the USA will result in a widespread outbreak and possibly become endemic, it is important to ascertain whether the widely distributed, sentinel domestic (sheep) and wildlife (WTD) ruminant species are susceptible. The aim of this study was to determine the susceptibility of WTD to the Northern European strain of BTV-8 to assess the potential disease risk to this wildlife species and to better understand the epidemiological role they would play, should an introduction occur.

2. Materials and methods

2.1. Virus inoculum

Virus (BTV-8/NET2007/01) was obtained from the Central Veterinary Institute of Wageningen University, Lelystad, the Netherlands. The BTV-8 inoculum was EDTA-blood harvested from Holstein Frisian cow NL441689187 from Bavel, Netherlands; the first detected case of BTV-8 after overwintering (GenBank GQ506451–GQ506460) (Backx et al., 2007). Briefly, cell fractions of the harvested EDTA-blood were washed three times with phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA), resuspended in the original volume, and stored in aliquots at −80 °C. Because the viral inoculum was infected blood and a not cell culture-adapted stock, it was necessary to use real time qRT-PCR to determine the relative titer, rather than cell culture titration methods. Mean threshold cycle (Ct) values (triplicate) were compared to a dilution series of known BTV stock to calculate RNA concentrations. Virus particle determinations, relative to RNA concentrations, were then calculated as described (Akita et al., 1992).

2.2. White-tailed deer (WTD)

Ten WTD fawns (2–14 days old) were obtained from a private deer facility in Missouri, USA. Fawns were raised and weaned at the Colorado State University (CSU), Animal Population Health Institute's Wildlife Research Facility, Fort Collins, CO, USA. Animals were raised according to a protocol approved by CSU's Institutional Animal Care and Use Committee (IACUC). Fawns were vaccinated subcutaneously (SC) with 1 ml Clostridium A toxoid (Novartis Animal Health, Greensboro, North Carolina, USA), dewormed with ivermectin (ProMectin®, Vedco, St. Joseph, MO, USA; 0.2 mg/kg, SC), and fenbendazole [Safe-guard®, Intervet/Schering-Plough, Summit, NJ, USA; 5 mg/kg orally (PO)] and given a 4-day course of sulfamethazine sodium (Sulmet®, Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA; 247.5 mg/kg day 1 and then 123.75 mg/kg days 2–4 once a day PO) for treatment and prevention of coccidiosis or other bacterial/parasitic infections. At one month, weaning began and all animals were given 3 ml Clostridium A toxoid as a SC booster. Additionally, they were given a 7-way clostridial vaccine (Ultrabac® 7; 2.5 ml SC) and tetanus toxoid (Colorado Serum, Denver, CO, USA; 1 ml SC), again dewormed and given anti-parasitic treatment with Ivermectin, sulfamethazine sodium, and fenbendazole. At approximately 9 months of age, the deer were sedated with an intramuscular combination of medetomidine hydrochloride (Wildlife Pharmaceuticals Inc., Fort Collins, CO, USA; 0.1–0.2 mg/kg), azaperone (Wildlife Pharmaceuticals Inc.; 0.1–0.2 mg/kg), and butorphanol tartrate (Wildlife Pharmaceuticals Inc.; 0.22–0.34 mg/kg) and transported to the

CSU, BSL-3 Animal Disease Laboratory, Fort Collins, CO, USA.

2.3. Animal experiment

Eight BTV-seronegative deer were sedated with xylazine (1–2 mg/kg) and injected with BTV-8/NET2007/01 bovine blood stock virus subcutaneously in the neck (1 ml) and inner left leg (1 ml). Two deer were sham inoculated with PBS to serve as uninfected controls and housed with infected animals to examine direct contact transmission. Body temperatures and clinical signs (depression, loss of appetite, oronasal discharge, respiratory distress, facial edema) were scored using a scale from 0–3 (0 = absence; 1 = mild; 2 = moderate; 3 = severe) (van Gennip et al., 2012) and recorded daily. Blood samples for cELISA and qRT-PCR assays were taken at specific time points from all deer. Two challenged animals were sedated with xylazine as above and euthanized with pentobarbital (IV) at the anticipated peak of viremia (day 8), two were euthanized for humane reasons due to deteriorating clinical condition (one each on day 12 and 15), and the remaining were held until day 28. During necropsy, gross pathological findings were noted and samples of liver, spleen, lung, heart, kidney, intestine, mandibular lymph node, mesenteric lymph node, and adrenal gland were collected and frozen for subsequent qRT-PCR testing, or fixed in 10% buffered formalin (Sigma–Aldrich), embedded in paraffin (Fisher Scientific, Houston, TX, USA) and sectioned (5 μm) for hematoxylin and eosin (Fisher Scientific) histopathological analysis. All animal manipulations were in compliance with the CSU IACUC guidelines. All work with infected animals was performed under BSL-3 containment conditions.

2.4. Real time PCR

Due to the unpassaged nature of this recent BTV-8 bovine virus isolate, cell culture-based titration assays were not feasible. Thus, qRT-PCR was utilized as the primary method to quantitate virus in the original inoculum and in blood and tissue samples. Viral RNA was extracted from blood using the MagMAX™ Blood RNA Isolation Kit (Ambion, Austin, TX, USA) according to manufacturer's instructions and stored in round bottom 96-well plates at −80 °C. For tissue RNA, 50 mg of tissue were individually homogenized by bead beating (TissueLyser, Qiagen, Germantown, MD, USA) in 500 μl PBS with two 4.5 mm stainless steel ball bearings. Total RNA was extracted from 100 μl of homogenate using the Total Viral RNA Isolation Kit (Ambion) according to manufacturer's instructions and stored in round bottom 96-well plates at −80 °C.

Quantitative real-time reverse transcriptase PCR (qRT-PCR) (Wilson et al., 2009) was performed on blood and tissue RNA samples, in triplicate (Applied Biosystems 7500, Foster City, CA, USA). Reactions contained 5 μl template and 20 μl master mix from Ag-Path ID™ RT-PCR reagents (Ambion) for 40 cycles. BTV-8 was detected using primer sequences specific for the S10 (NS3) and M5 (NS1) genes which are highly conserved within the serogroup and highly expressed. Primer sequences were TTAAGCCTCCTAGGTCACTTTTCAA (forward), AAAGCTGCATTCGCATCGT (reverse), with CACATCATCACGAAACGCTTCTGCG-5′CFO 560 and 3′BHQ-1 (probe) for S10; GATTGCTTCACGGCCTCAT (forward), TTGGCAAAGGAGGCAATGT (reverse), with TGCACCCCGCACCGCTTC-5′CFO 560 and 3′BHQ-1 (probe) for M5. Additionally, the house-keeping gene β-actin was used as an internal, same tube control using primers as previously described (Moniwa et al., 2007). As above for the inoculum, mean Ct values (triplicates) of the deer samples were compared to known BTV stock concentrations to calculate RNA concentrations. Virus particle determinations relative to RNA concentrations were calculated as described (Akita et al., 1992) and are reported as $\log_{10}$ particles/ml of blood or tissue homogenate.

2.5. Detection of infectious virus

Due to the non-adapted nature of the BTV-8 inoculum to cell culture systems, blind passages of virus from blood samples were performed to obtain cytopathic effect (CPE) and confirm the infectious virus as the original viral inoculum by qRT-PCR and sequence analysis. Day 8 blood samples (1 ml each) from all inoculated deer were sonicated (Q700; Qsonica, Newtown, CT, USA) for 3 min with 5 s pulses at 100 mA and centrifuged for 10 min at 10,000 × *g*. Cleared supernatants (500 μl) were used to inoculate T-25 flasks (Corning, Corning, NY, USA) containing baby hamster kidney (BHK; ATCC, Manassas, VA, USA) cell monolayers grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 10% FBS (Sigma–Aldrich) and 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Sigma–Aldrich). Flasks were incubated at 37 °C for 5–7 days and examined for CPE. CPE flasks were freeze–thawed and 2 ml used to inoculate fresh cells twice more for a total of three blind passages. A 1 ml aliquot was used from each passage for RNA extraction and BTV-8 qRT-PCR as described above. Additionally, viral RNA was extracted, as above, from the blood of deer #15 (euthanized day 8) and the VP2 gene, which confers serotype identity and has the most variable genomic region among the serotypes (Roy, 2001), was sequenced and compared with the original inoculum (Maan et al., 2010; van Gennip et al., 2012). Four forward and three reverse primers were used for full gene coverage. Forward primers were VP2-1F ATGGAGGAGCTAGCGATTCCGAT; VP2-620F AAATGCGGAACGTGCATCAACGAG; VP2-1356F TGCGATCTGTTCCCTGAGGATGAA; and VP2-1972F AACACCCAACAATTGATCCCAGCG. Reverse primers were VP2-2903R CTATACATTGAGCAGCTTAGTTA; VP2-1837R CGCCTTGAAACAATCCCTGCGTAA; and VP2-643R CTCGTTGATGCACGTTCCGCATTT.

2.6. Serological analysis

Serum samples from experimentally infected deer were tested for BTV antibody using a previously published antigen capture enzyme-linked immunosorbent assay (cELISA) (Mecham and Wilson, 2004). Briefly, the wells of Immunlon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA, USA) were coated overnight at 4 °C with

polyclonal rabbit anti-BTV serum. Antigen was then captured in the wells by addition of baculovirus expressed BTV VP7 protein. Test serum samples, in triplicate, were diluted 1:5 in PBS containing 0.1% bovine serum albumin (BSA; Sigma–Aldrich) in the microtiter plate wells (final volume of 50 μl/well), followed by the addition of 50 μl/well of diluted mouse monoclonal antibody specific for VP7 of BTV. This was followed by sequential reactions with biotinylated goat anti-mouse antibody (Biogenex, Fremont, CA, USA) and peroxidase-conjugated streptavidin (Biogenex). Measurement of optical density (OD), following addition of ortho-phenylene diamine (OPD) substrate (Sigma–Aldrich), was used to calculate percent inhibition (PI): $PI = 100 - (OD_{492\,nm}$ test serum/$OD_{492\,nm}$ negative serum) $\times 100$. Pre-infection sera were used to determine the OD negative serum values. Sera were also tested for antibody to the related serologically cross-reactive epizootic hemorrhagic disease virus (EHDV), as determined using an antigen-capture cELISA to that virus (Mecham and Wilson, 2004).

3. Results

3.1. Clinical observations

Clinical signs in WTD were scored based on severity (van Gennip et al., 2012) and included depression, loss of appetite, nasal discharge, respiratory distress, facial edema, conjunctivitis, conjunctival erythema, excessive salivation, and elevated rectal temperatures (Table 1; Fig. 1). Clinical disease peaked from 8 to 15 days post inoculation (dpi) and was typically cleared by 21 dpi in surviving animals. Clinical signs often associated with BT that were not seen in the experimentally infected WTD included oronasal lesions, coronitis, and cyanotic (blue) tongue.

Two randomly chosen deer (#15 and #23) were euthanized during the anticipated peak of viremia (8 dpi) to examine virus distribution in tissues. The livers of both deer had mild, chronic portal hepatitis and multifocal chronic interstitial nephritis and their lungs had mild, diffuse mononuclear interstitial pneumonia. Additionally, two deer (#21 and #6) had to be euthanized during the peak of their clinical disease for humane reasons. Deer #21, euthanized at 12 dpi, had severe nasal discharge, excessive salivation, and respiratory distress. Histopathological findings included mild, chronic portal hepatitis and mild, diffuse mononuclear interstitial pneumonia. Deer #6, euthanized at 15 dpi, had severe depression, loss of appetite, nasal discharge, excessive salivation, respiratory distress, facial edema, low body temperature, abnormal gait and loss of balance. Gross pathological findings of these two deer was mild but consistent with BTV infection and included hemorrhages in the spleen, kidney, and intestine, interstitial edema in the lungs, and mild accumulation of fluid in the pericardium (deer #6 only). Histopathological findings included mild, chronic portal hepatitis, mild white pulp lymphocytolysis and lymphoid depletion of the spleen, and severe necrotizing bronchopneumonia. No gross pathology was seen in deer euthanized at 28 dpi ($n = 4$), the end of the experiment.

Clinical signs of disease were not observed in the two sham inoculated negative control deer which were housed with the inoculated deer. Additionally, no BTV RNA was detected in blood or tissue samples confirming, as expected, that BTV-8 was not transmitted between WTD by direct contact.

3.2. Molecular analysis

The BTV-8/NET2007/01 washed blood cell inoculum was determined to be 5.78 $\log_{10}$ particles/ml (6×10^5/ml) by qRT-PCR and RNA/virus particle determinations. Thus, with the inoculation of 1 ml at two sites, the deer received a total of 6.08 $\log_{10}$ particles (1.2×10^6). Viremia in inoculated deer, as detected by qRT-PCR, started as early as 3 dpi, peaked from day 7 to 15 and persisted through day 28 in 3 of 4 surviving deer (Fig. 2). Blood virus titers in the two deer euthanized on day 8 (#15 and #23) was 6.40 and 6.56 $\log_{10}$ particles/ml, respectively (Fig. 2A). Peak viremia in the two deer euthanized during the peak of clinical disease (#21 and #6) was 7.4 and 8 $\log_{10}$ particles/ml on 10 and 12 dpi, respectively (Fig. 2B). The highest viremia detected at the end of the study (day 28) was 6.13 $\log_{10}$ particles/ml (deer #16; Fig. 2C).

As expected for a viremic infection, the distribution of BTV-8 in tissues as detected by qRT-PCR was widespread. In the two deer euthanized on day 8 (#15 and #23) viral RNA was detected in all tissue homogenates tested (liver, spleen, lung, heart, kidney, intestine, mandibular lymph node, mesenteric lymph node, and adrenal gland). The highest titers ($\log_{10}$ particles/ml of homogenate) for #15 and #23 were seen in the heart (7.64 and 5.73), lung (5.57 and 7.32),

Table 1
Peak clinical signs from individual deer during challenge with serotype 8 of bluetongue virus.

Deer	Depression	Loss of appetite	Oronasal discharge	Respiratory distress	Facial edema	Temperature (°C)[b]	Peak viremia ($\log_{10}$/ml)[c]
6	3[a]	3	2	1	3	40.6	7.0
7	2	3	0	0	2	39.6	7.0
14	0	3	1	1	2	40.1	6.4
15	0	0	0	0	0	41.2	6.4
16	0	3	0	0	0	39.6	6.7
20	2	3	0	2	1	40.8	6.7
21	2	3	3	3	3	40.6	6.4
23	0	0	0	0	0	40.2	6.6

[a] Peak clinical signs using a scale from 0 to 3: 0 = Absent, 1 = Mild, 2 = Moderate, 3 = Severe condition.
[b] Rectal temperatures in Celsius, average daily temp of control deer 39 °C.
[c] Peak viremia as calculated by qRT-PCR.

Fig. 1. Clinical bluetongue virus serotype 8 disease in white-tailed deer. (A) facial edema, excessive salivation, nasal discharge, conjunctival erythema (#21, 9 dpi; (B) anorexic, abnormal gait, loss of balance (#6, 15 dpi); (C) facial edema, conjunctival erythema (#21, 12 dpi; (D) respiratory distress as indicated by open mouth breathing (#21, 12 dpi).

and liver (6.05 and 6.84), respectively (Fig. 3A). For the two deer euthanized during the peak of their clinical disease (#21 at 12 dpi and #6 at 15 dpi), viral RNA was detected in all tissues tested with the highest titers ($\log_{10}$ particles/ml of homogenate) seen in the spleen in both deer (9.62 and 8.98, respectively) (Fig. 3B). At day 28, there was no consistent pattern of viral tissue distribution and related viremia in the four surviving BTV-8 inoculated deer. Viral RNA was detected in only a single tissue in the three remaining viremic deer; the spleen of #7 (5.47), the lung of #14 (5.18) and the mesenteric lymph node of #16 (7.40 $\log_{10}$ particles/ml) (Fig. 3C). Whereas, the deer with no detectable viremia from day 21 through 28 (deer #20) showed the greatest distribution of persisting viral RNA with detection in all tissues tested except the intestine.

3.3. Virus isolation

Infectious virus was isolated from the blood of all eight inoculated deer in CPE flasks and confirmed as BTV-8 by NS3 and NS1 qRT-PCR (data not shown). Additionally, a 2841 nucleotide sequence of the BTV-8 serotype specific VP2 serotype specific gene obtained from the blood of deer #15 (8 dpi) had 99.5% identity (100% similarity) to the original BTV-8/NET2007/01 inoculum.

3.4. Serological analysis

Antibody specific to BTV was detected by the antigen-capture cELISA in the serum of all experimentally infected WTD (Fig. 4). Sera were scored positive for BTV antibody if the PI was $\geq$50%, since a lower PI could increase the chance of including false positives. Antibody responses in the WTD were detected as early as 8 dpi and as late as 12 dpi, with the majority of animals showing at least 70% cELISA inhibition by 10 dpi. None of the experimental animals were positive for antibody to the related serologically cross-reactive EHDV (data not shown).

4. Discussion

The recent incursion of BTV-8 into Northern Europe and the UK has highlighted the risk that countries face for accidental or intentional introductions of serotypes with

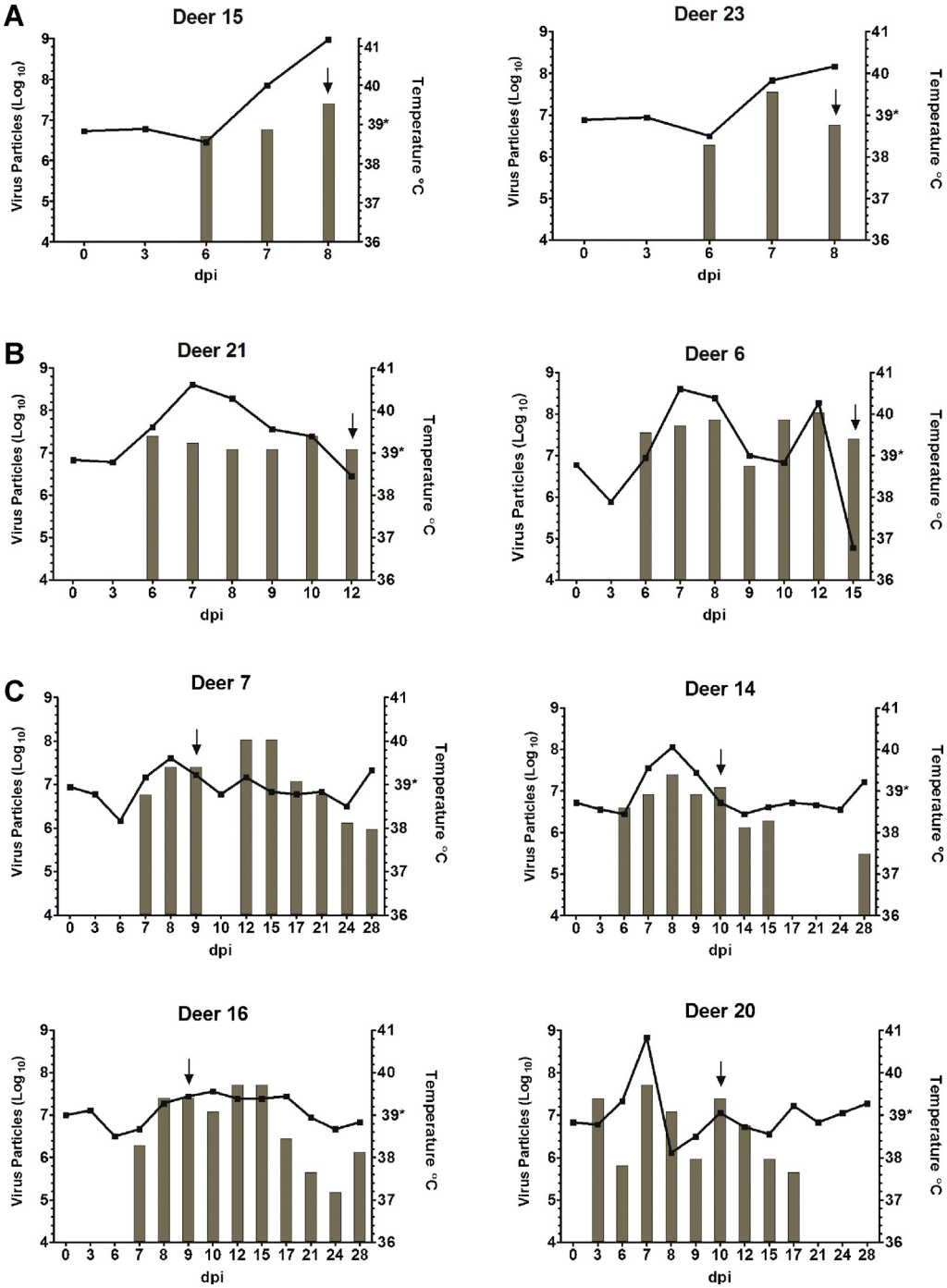

Fig. 2. Rectal temperatures (line) and viremia (bars) as detected by qRT-PCR of white-tailed deer inoculated with bluetongue virus serotype 8. Day of peak clinical disease for each animal is indicated with an arrow. (A) Deer euthanized at 8 dpi (#15 and #23) during the expected peak of viremia; (B) deer euthanized at 12 dpi (#21) and 15 dpi (#6) during the peak of clinical disease; (C) deer euthanized at 28 dpi. Normal temperature 39 °C (asterisk).

unknown virulence into naïve domestic and wild ruminant herds. With varying virulence, and little to no cross protection between outbreak isolates, the 26 serotypes of BTV are a constant disease threat to North American livestock and wildlife. Susceptible hosts and *Culicoides* competent for the endemic BTV serotypes are found throughout the country which results in an environment very favorable for the already identified (Ostlund, 2010) or new exotic isolates to become established upon introduction *via* infected midge or animal.

Because BTV vectors (*Culicoides* spp.) feed on both wild and domestic ruminants, the wildlife-livestock interface is

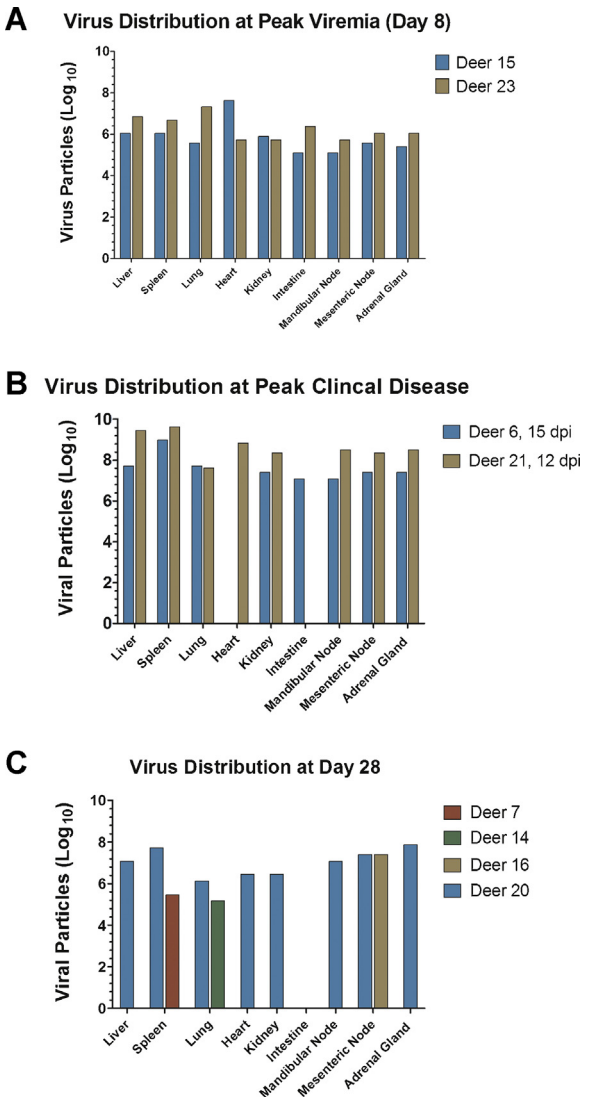

Fig. 3. Distribution of bluetongue virus serotype 8 in tissues of experimentally infected white-tailed deer as detected by qRT-PCR. (A) Deer euthanized at 8 dpi (#15 and #23) during the peak of viremia; (B) deer euthanized at 12 dpi (#21) and 15 dpi (#6) during the peak of clinical disease; (C) deer euthanized at 28 dpi. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a critical factor in the epidemiology of outbreaks and risk assessment in terms of viral amplification and geographic distribution (Drolet et al., 1990; Garcia et al., 2009; Garcia-Bocanegra et al., 2011). This is the first report of experimental infection of North American WTD with the European strain of BTV-8. Our findings demonstrate that BTV-8 infection produces moderate to severe clinical disease and that during the peak of both viremia and clinical disease, virus is widespread in tissues. At peak clinical disease (deer #6 and #21), titers of virus as detected by qRT-PCR were higher in some tissues than in the blood. This was especially the case for deer #21 with from 3.5 (lung) to 344 (spleen) times more virus in 1 ml of tissue homogenate than 1 ml of blood at the time of

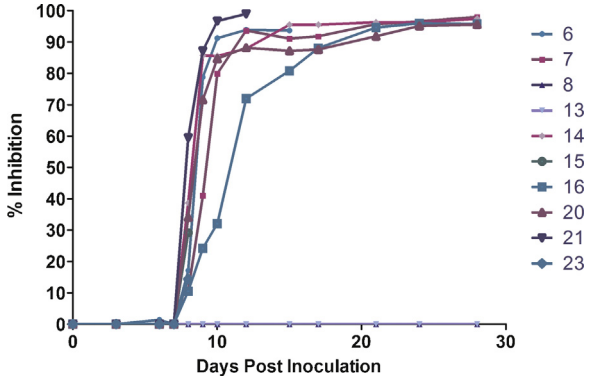

Fig. 4. Antibody response as detected by competitive ELISA in serum of white-tailed deer inoculated with bluetongue virus serotype 8. Deer #15 and #23 were euthanized on day 8; deer #21 on day 12; deer #6 on day 15; deer #7, 14, 16, and 20 on day 28. Deer #8 and #13 (negative controls) were euthanized on day 28. Sera were scored positive for BTV antibody if the PI was ≥50%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

necropsy. These results suggest active virus replication in several tissues at the peak of clinical disease.

Of the four surviving deer at day 28, three had detectable viremia. However, fluctuations during the 28-day study, as well as animal to animal variation were seen. In deer #20, viral RNA persisted in all tissues except the intestine without a corresponding viremia. A drop in viremia was also seen in deer #14 and #16 from 17 to 24 days followed by a recovery on day 28. This trend may suggest a cyclical viremia where virus or viral RNA persisted in some tissues, but was shed and cleared in peripheral blood intermittently after peak clinical disease. Oscillating patterns of BTV viremia, as detected by qRT-PCR, have also been shown in sheep (Batten et al., 2012), cattle (Di Gialleonardo et al., 2011) and camels (Batten et al., 2011). A longer term study would be required to ascertain this trend and whether it may have diagnostic implications where virus persists in the absence of viremia. It is possible that immune responses may influence tissue and blood clearance of virus, but the mechanistic basis for this variable immunity is not understood.

Although seroprevalence studies of wildlife in European outbreak areas have shown the presence of BTV-8 specific antibody in red (*Cervus elaphus*), fallow (*Dama dama*) and roe (*Capreolus capreolus*) deer, little to no disease was observed suggesting these wildlife species may have acted as subclinical reservoirs (Linden et al., 2008; Conraths et al., 2009; Garcia et al., 2009; Falconi et al., 2011; Garcia-Bocanegra et al., 2011). This was substantiated in experimental BTV-8 infections of red deer (Lopez-Olvera et al., 2010; Lorca-Oro et al., 2012) and is similar to seroprevalence reports and field observations for BTV-4 in Spain (Ruiz-Fons et al., 2008; Rodriguez-Sanchez et al., 2010; Garcia-Bocanegra et al., 2011).

In general, North American WTD appear to be much more susceptible to BTV infection and development of clinical disease (Howerth et al., 1988; Parsonson, 1990; Quist et al., 1997; Stallknecht and Howerth, 2004) than Eurasian deer species as described above. Prior to this study, however, it was not clear whether infection of WTD

with the European strain of BTV-8 would result in subclinical disease as seen in Europe, clinical disease similar to North American domestic serotypes, or more severe disease levels. Falconi et al. (2011) reported that BTV serotype 8 had previously been shown to cause clinical disease in WTD. Their referenced report (Vosdingh et al., 1968) actually states that BTV-8 "strain" was used, not BTV serotype 8. This was the California BTV strain 8 isolate from USDA-ARS, which subsequently typed as BTV-10 (Yu et al., 1987). More recent reports using this strain refer to it as "CA-8 BTV-10" or "BTV-10; BT-8 strain" (Wilson et al., 1990). Thus, this is the first report of experimental infection of North American white-tailed deer with BTV serotype 8.

For this study, the BTV-8 inoculum used was infected bovine blood from the first detected 2007 case of BTV-8 in the Netherlands after overwintering. Although more difficult to work with in some respects, recent isolates from naturally infected animals are desirable for animal infection studies, as there can be some degree of confidence that cell passage-associated mutations and *in vitro* fitness selection have not occurred and will not affect the resulting infectious process. However, the lack of being cell culture adapted makes cell culture-based titrations of blood and tissues impossible. In terms of epidemiological predictions, care should always be taken in extrapolating qRT-PCR quantitation and subsequent particle calculations to infectious virus titers and duration of viremia.

Understanding the degree to which our wildlife and livestock species are susceptible to BTV-8 is critical in determining relevant epidemiological assessments and risk analyses for the USA. WTD are the wildlife species most often affected by endemic serotypes of BTV, and are therefore the likely wildlife species to play a role in the ability of BTV-8 to become established. Although more clinical disease was observed in WTD compared to that seen with both natural (Conraths et al., 2009; Falconi et al., 2011; Garcia-Bocanegra et al., 2011) and experimental (Lopez-Olvera et al., 2010; Lorca-Oro et al., 2012) infection of Eurasian deer species, it was consistent with BT disease levels previously reported for North American domestic serotypes (Hoff and Trainer, 1978; Stallknecht et al., 1995; Quist et al., 1997). Our findings suggest that should BTV-8 be introduced in the USA, WTD would be expected to act as clinical disease sentinels and survival rates would be sufficiently high to act as significant virus amplifying reservoirs for subsequent transmission of virus by *Culicoides* spp. to livestock and other wildlife species.

Conflicts of interest

None.

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