

Experimental infection of Holstein cows and calves with EHDV-7 and preliminary evaluation of different inoculation methods

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Summary

Infection of cattle with Epizootic haemorrhagic disease (EHD) virus (EHDV) is frequently subclinical, yet reports of disease have increased in recent years. In 2006, a widespread EHDV-7 epidemic caused disease and economic loss in the Israeli dairy industry. In this study, the main objective was to infect cattle with EHDV-7 and replicate disease observed in Israel during 2006. Two infection studies were performed. Experiment 1, 4 cows inoculated with intradermal (ID) and subcutaneous (SC) injections with an EHDV-7 blood inoculum. Experiment 2, 6 calves inoculated using 1 of the following 3 methods (2 calves/method): (1) mammalian cell culture supernatant by ID and SC injection; (2) culture supernatant by ID, SC, and intravenous injection; and (3) bite transmission from *Culicoides sonorensis*. Further, during experiment 2, *C. sonorensis* were fed on 4 infected calves (18 days post-inoculation) and processed for virus isolation 10 days later in order to evaluate infectivity of low-titer viraemia. Three cows had detectable viraemia and all 4 seroconverted. No clinical signs were observed. All 6 calves developed viraemia, peaking 7-10 dpi and all calves seroconverted. No differences in virus kinetics were observed between the inoculation groups. Calves in group 2 had transiently elevated rectal temperatures but no other clinical abnormalities were observed. The 124 midge pools processed after feeding on calves with low-titer viraemia were virus isolation negative. Detectable viraemia was more consistent in calves than adult cows. This study demonstrates US-origin cattle are susceptible to EHDV-7 infection by multiple inoculation methods; however, as reported in other studies, the disease was not replicated experimentally.

Infezione sperimentale di vacche e vitelli Holstein con virus della Malattia emorragica epizootica (EHDV-7): valutazione preliminare dei diversi metodi di inoculazione

Parole chiave

Bovini,
Culicoides sonorensis,
Infezione sperimentale,
Malattia emorragica
epizootica,
Metodo di inoculazione,
Sierotipo 7 del
virus della Malattia
emorragica epizootica
Vettore.

Riassunto

Sebbene negli ultimi anni le forme cliniche siano aumentate, l'infezione dei bovini con il virus della Malattia emorragica epizootica (EHDV) è per lo più subclinica. In Israele, nel 2006, il sierotipo 7 dell'EHDV (EHDV-7) è stato responsabile di gravi forme cliniche nei bovini e considerevoli perdite economiche nel settore lattiero-caseario. Lo studio ha avuto l'obiettivo di riprodurre attraverso infezione sperimentale la forma clinica osservata in Israele nel 2006 nei bovini. Esperimento n. 1: 4 vacche sono state infettate per via intradermica (ID) e sottocutanea (SC) con sangue infetto con EHDV-7. Esperimento n. 2: 6 vitelli in gruppi di 2 sono stati inoculati con: metodo 1, surnatante di colture cellulari di mammifero per via ID e SC; metodo 2, surnatante di colture per via ID, SC ed endovenosa; metodo 3, trasmissione mediante puntura di *Culicoides sonorensis*. In aggiunta, durante l'esperimento n. 2, esemplari di *C. sonorensis* sono stati messi a contatto con i 4 vitelli viremici (18 giorni dopo l'infezione)

su cui hanno effettuato il pasto di sangue e, dopo 10 giorni, processati per rilevare la presenza del virus e valutare l'infettività e il titolo virale. In 3 vacche è stata evidenziata viremia mentre in tutte e 4 è stata dimostrata sierconversione. Non sono stati osservati sintomi. Tutti i vitelli hanno sviluppato viremia che ha avuto il suo acme a 7-10 giorni dall'inoculazione, tutti gli animali hanno sierconvertito. Non sono state osservate differenze nella cinetica del virus tra i gruppi infetti. I vitelli del gruppo 2 hanno mostrato transitoriamente elevate temperature rettali, ma non sono stati osservati altri segni clinici. Nei 124 pool di *Culicoides* esaminati dopo il pasto di sangue su vitelli viremici non è stato possibile isolare il virus. Titoli viremici sono stati rilevati con maggior frequenza nei vitelli che nei bovini adulti. Questo studio dimostra che i bovini di origine statunitense sono suscettibili alle infezioni da EHDV-7 attraverso diverse vie d'inoculazione. Tuttavia, come riportato in altri studi, non è stato possibile riprodurre sperimentalmente la malattia.

Introduction

Epizootic haemorrhagic disease (EHD) viruses (EHDV) are double-stranded RNA viruses in the genus *Orbivirus* transmitted by *Culicoides* biting midges to a variety of wild and domestic ruminant hosts. There are 7 proposed EHDV serotypes (Anthony *et al.* 2009), which are generally considered to exist in tropical and temperate regions of the world supporting populations of competent vectors (Savini *et al.* 2011). Despite this apparent broad virus distribution, published reports of EHD are historically infrequent outside of North America, where the EHDV-1, EHDV-2, and EHDV-6 are well-known pathogens of white-tailed deer (WTD, *Odocoileus virginianus*) and other wild ruminants (Howerth *et al.* 2001). However, recent reports of EHDV-associated disease in cattle in certain parts of the world (Bréard *et al.* 2004, Temizel *et al.* 2009, Yadin *et al.* 2008) and the documentation of production loss in dairy cattle (Kedmi *et al.* 2010b) have raised questions regarding the significance of EHDV infection in cattle, as well as the role of cattle in the epidemiology of EHD.

Although EHDV infection in cattle is relatively common in certain parts of the world, clinical disease is generally absent (St. George 1983, Odiawa 1985, Metcalf 1992, Aradaib 1994). In fact, 4 of the 7 EHDV serotypes (5, 6, 7, and 8) were all originally isolated from asymptomatic sentinel cattle in Australia (St George *et al.* 1983). The only consistent exception has been EHDV-2 (strain Ibaraki), which has caused periodic outbreaks of EHD in Japanese cattle since 1959 (Omori *et al.* 1969). The disease, often referred to as Ibaraki disease, is characterised by transient fever, stomatitis, and difficult deglutition (Omori *et al.* 1969). Outside of far East Asia, confirmed reports of disease in cattle infected with EHDV from 1950-2000 are relatively uncommon (Metcalf *et al.* 1992, Barnard *et al.* 1998), but appear to have been more frequent since 2000, including EHD outbreaks in cattle from Israel (Yadin *et al.* 2008), Réunion

Island (Bréard *et al.* 2004, Sailleau *et al.* 2011), Morocco (EFSA 2009), Algeria (EFSA 2009), Turkey (Temizel *et al.* 2009), Tunisia (Savini *et al.* 2011), Jordan (Savini *et al.* 2011), and the United States (Ostlund 2009, Dudley 2013, Rodman 2013).

During a 2006 EHDV-7 outbreak in Israel, the infection was widespread and the disease was reported in 105 cattle herds (Yadin *et al.* 2008). Decreased appetite, transient fever, weakness, and stiff gait were commonly observed, although the most common consequence of infection was a drop in milk production (Yadin *et al.* 2008, Kedmi *et al.* 2010b). Occasionally, more overt clinical signs were observed, such as nasal discharge, ptialism, conjunctival hyperaemia, palpebral oedema, hyperaemia of oral mucosa, and occasional oral petechiae and erosions. Yet, despite the observed morbidity, serosurveys indicate that subclinical infection was also common during the outbreak (Kedmi *et al.* 2010a). Mortality was < 1%, but the production losses resulted in significant economic consequences for the Israeli dairy industry (Kedmi *et al.* 2010b).

Although Bluetongue virus (BTV) has been present in Israel for decades, EHDV had never been documented in Israel and the cattle population was considered to have been immunologically naïve to EHDV prior to 2006 (Kedmi *et al.* 2010a). Cattle herds in the European Union are also considered to be naïve although they are considered at risk for potential EHDV incursions in the future, underscoring the need for a better understanding of EHD in cattle (EFSA 2009). Despite EHDV-7 being exotic to the United States (US), cattle in parts of the US are commonly exposed to endemic EHDV serotypes (Metcalf *et al.*, 1992; Odiawa *et al.*, 1985). Thus, although not a truly immunologically naïve population, the susceptibility of US cattle to EHDV-7 infection and disease is unknown. The objective of this study was to determine the susceptibility of US-origin Holstein cattle to EHDV-7 infection and to replicate clinical disease observed in Israel during

the 2006 epidemic. Previous studies demonstrated that WTD are susceptible to infection and severe disease with EHDV-7 (Ruder *et al.* 2012a). It has also been shown that *Culicoides sonorensis* is a competent vector (Ruder *et al.* 2012b), confirming that potential ruminant and vector hosts for this exotic EHDV are present in the US. Here we describe two studies: (1) experimental infection of Holstein cows with EHDV-7, and (2) a follow-up experimental infection of Holstein calves to preliminarily evaluate the effect of different inoculation methods on infection outcome with the goal of replicating disease observed in the field. A secondary objective of the second study was to evaluate the infectivity of cattle with low-titer viraemia to *C. sonorensis*, a North American vector of EHDV.

Materials and methods

Animals and *Culicoides*

Five, mature (> 4 years) Holstein cows (400-600 kg) from a dairy farm in Oglethorpe County, Georgia (US) and 7, 2 month-old Holstein calves (70-90 kg) from Monroe County, New York (US, Thomas Morris, Inc.) were obtained. Additionally, three 1-4 week-old WTD were acquired from Athens-Clarke County (Georgia, USA) (Whitehall Deer Research Facility, University of Georgia) and hand-raised until 4-6 months-old. All animals were negative for precipitating antibodies to EHDV and BTV using agar gel immunodiffusion (AGID) test kits (Veterinary Diagnostic Technology, Inc, Wheatridge, CO, USA), and for neutralizing antibodies against EHDV-1 and EHDV-2 using previously described methods (Stallknecht *et al.* 1995). All procedures described in these studies were approved by the University of Georgia's Institutional Animal Care and Use Committee. Adult *C. sonorensis* (Jones and Foster 1974) were obtained from the Arthropod-Borne Animal Diseases Research Unit (USDA-ARS, Manhattan, KS, USA). Midges were allowed to emerge in Kansas and one-day-old adults were shipped to Georgia overnight by air and held at room temperature ($21 \pm 1^\circ\text{C}$) with distilled water for 1-2 days before use.

Virus and inoculum

The virus used in these studies was provided by the Pirbright Institute (Woking, Surrey, UK), where it was originally isolated from the blood of a cow using an embryonated chicken egg and subsequently passaged once on *Culicoides* cell line (KC cells) (Wechsler *et al.* 1989) and identified as EHDV-7 (Pirbright virus collection number, EHDV ISR2006/04). To comply with the permit

requirements for importing the virus into the US, the isolate was serially passaged 5 times on baby hamster kidney (BHK) cells (American Type Culture Collection, Manassas, VA, USA) at the Pirbright Institute. Live-animal safety testing of the virus stock was then performed at the USDA's Plum Island Animal Disease Research Center (Plum Island, NY, USA) to test for foot and mouth disease virus before shipment to the University of Georgia. The titer of the imported virus stock was $10^{6.2}$ median tissue culture infective doses (TCID₅₀/ml) (Reed and Muench 1938). In Georgia, this virus stock was initially passaged through a single WTD (Ruder *et al.* 2012a). Briefly, a 4-month-old WTD was inoculated by subcutaneous (SC, 1 ml) and intradermal (ID, 1 ml) injections; on 6 days post-inoculation (dpi) whole blood was collected and a blood inoculum prepared as previously described (Quist *et al.* 1997b). This WTD developed mild disease (*e.g.*, fever, lethargy, lymphopenia, erythema of non-haired skin). The virus titer of this WTD blood inoculum was $10^{4.3}$ TCID₅₀/ml.

Cow trial

To prepare a virus inoculum for the Holstein cow trial, the WTD blood inoculum described above was used to inoculate a second WTD fawn by SC (5 ml) and ID (5 ml) injection. This animal developed moderate-to-severe disease as described previously (Ruder *et al.* 2012a); on 6 dpi, whole blood was collected and another blood inoculum prepared ($10^{5.1}$ TCID₅₀/ml), which was used to inoculate the cows. Prior to infecting the second WTD, whole blood was collected in order to prepare a sham blood inoculum for the negative control cow.

Calf trial

For the experimental infection of Holstein calves, a single passage cell culture-based inoculum was used. To prepare the inoculum, the original WTD blood inoculum described above was used to infect a group of WTD as part of a previous study (Ruder *et al.* 2012a). *Culicoides sonorensis* were then infected with EHDV-7 after taking a blood meal from these WTD at peak viraemia before transmitting the virus to another WTD (Ruder *et al.* 2012b). The virus was subsequently isolated on BHK cells from the lung and an inoculum was prepared as previously described (Gaydos *et al.* 2002a). The virus titer of the inoculum after single passage in BHK cells was $10^{7.0}$ TCID₅₀/ml. Depending on inoculation method in the calves, this virus inoculum was then used to either inoculate calves directly or spike blood for an infectious blood meal for *C. sonorensis*, which were then held in incubation and later used to inoculate calves.

Experimental design

Cow trial

During October 2010, 4 cows and the positive control deer were inoculated with 10 ml (5 ml SC and 5 ml ID, $10^{6.1}$ TCID₅₀) of the blood inoculum over the lateral cervical region. The negative control cow was similarly sham-inoculated. Animals were visually monitored twice daily and given a cursory physical exam, including rectal temperature, once daily 0-12 dpi and on 18 dpi. At approximately the same time on 0, 3, 5, 7, 10, 12, and 18 dpi, blood was collected for virus isolation and titration, serology, complete blood count (CBC), activated partial thromboplastin time (APTT), and prothrombin time (PT). All clinical pathology assays were performed using standard protocols at the Clinical Pathology Laboratory (Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA, US). Cows were euthanized on 18 dpi with intravenous sodium pentobarbital for post-mortem examination and virus isolation from selected tissues.

Calf trial

Following the results of the cow trial, 2-month-old Holstein calves were obtained from New York during January 2012. These animals had never been previously exposed to *Culicoides* spp., and it is extremely unlikely that this herd had previous *Orbivirus* exposure at this Northern latitude. Three inoculation methods were used (2 calves/method) with the goal of inducing clinical signs: group 1: combination of ID and SC injections of BHK cell culture supernatant (1.5 ml/route, $10^{7.12}$ TCID₅₀); group 2: combination ID, SC, and IV injections of BHK supernatant (0.67 ml/route, $10^{7.12}$ TCID₅₀); and group 3: bite transmission by laboratory infected *C. sonorensis*. A negative control calf received non-infected BHK supernatant similar to group 2. For group 3 animals inoculated by infected *C. sonorensis*, midges were given an infectious blood meal through a parafilm membrane stretched over the reservoir of an artificial feeding device as previously described (Bernardo and Cupp 1986). After a 10 day incubation period at 26°C, surviving midges were transferred to feeding cages and allowed to take a blood meal from the 2 calves in group 3, as previously described (Ruder et al. 2012b). Those midges that took a blood meal were then placed into virus transport media and processed individually for virus isolation and titration. Based on previous studies with BTV, the midges with virus titer $\geq 10^{2.7}$ TCID₅₀/midge were considered potentially competent (Jennings and Mellor 1987). All calves were monitored daily with a brief physical examination and rectal temperature,

and blood was collected on 0, 1 (group 2 only), 3, 5, 7, 10, 13, and 18 dpi for virus isolation and titration, serology, and CBC. In addition, in order to preliminarily evaluate the potential for cattle with low titer viraemia ($< 10^{2.3}$ TCID₅₀/ml) to infect *Culicoides*, *C. sonorensis* were allowed to take a blood meal from calf 77, 80, 84, and 89 on 18 dpi. After feeding on calves, midges were sorted according to feeding status and blood engorged midges were held at 26°C and on 10 days post-feeding (dpf) were placed into virus transport media in groups of 5 for virology. Following *Culicoides* feeding on 18 dpi, calves were euthanized for post-mortem examination and virus isolation attempted from selected tissues.

Virology and serology

Virus isolation and titration from blood was performed using cattle pulmonary artery endothelium (CPAE, American Type Culture Collection) cells as described (Quist et al. 1997b). Cultures were maintained and monitored as previously described (Ruder et al. 2012a) and the virus isolates were confirmed as EHDV-7 by real time polymerase chain reaction (RT-PCR) using published primers (Maan et al. 2010). *Culicoides sonorensis* were processed for virology as previously described (Ruder et al. 2012b), with the exception that virus isolation (pools of five) was performed on those midges that fed on calves on 18 dpi. For the virus titration format used for both blood and *Culicoides*, the minimum detectable titer was $10^{2.3}$ TCID₅₀/ml or /midge. Virus isolation from tissues was performed as described (Ruder et al. 2012a). Serum samples were tested for precipitating antibodies against EHDV using AGID test kits (Veterinary Diagnostic Technology, Inc, Wheat Ridge, CO, USA) according to manufacturer instructions. Beginning on the time point prior to the first AGID-positive samples, serum was tested for the presence of neutralizing antibodies by serum neutralization test and antibody titers were determined as previously described (Stallknecht et al. 1995), except for the use of CPAE cells in place of BHK cells.

Results

Cow trial

Viraemia and serological profiles are shown in Table I. Three cows developed a low-titer viraemia ($< 10^{2.3}$ TCID₅₀/ml) beginning 3 dpi and lasting through 10 dpi (n = 2) or 18 dpi (n = 1), 1 cow never had a detectable viraemia. Precipitating and neutralizing antibodies were detected by 7-10 dpi, and by 18 dpi, all 4 animals had a neutralizing antibody titer of 160. No clinical abnormalities, including fever ($\geq 39.5^\circ\text{C}$), were observed in any of the cows during the study.

Table I. Comparison of viremia profiles and serologic responses of Holstein cattle experimentally infected with serotype 7 of the Epizootic haemorrhagic disease virus (EHDV-7).

Animal ID	Test	Day post-inoculation						
		0	3	5	7	10	12	18
24	VT ^a	–	–	–	–	–	–	–
	SNT ^b			<10	20	20	40	160
95	VT	–	<2.3	<2.3	<2.3	<2.3	<2.3	<2.3
	SNT			<10	<10	<10	20	160
10	VT	–	<2.3	<2.3	<2.3	<2.3	–	–
	SNT			<10	10	20	80	160
25	VT	–	<2.3	<2.3	<2.3	<2.3	–	–
	SNT			<10	10	40	80	160
NC ^c	VT	–	–	–	–	–	–	–
	SNT			<10	<10	<10	<10	<10
PC ^d	VT	–	3.63	died				
	SNT			<10				

^a VT = virus titration, reported as log₁₀ median tissue culture infective doses per ml. Minimum detectable titer of 10^{2.3} TCID₅₀/ml.

^b SNT = serum neutralizing titer to EHDV-7, reported as reciprocal of the dilution.

^c NC = negative control cow.

^d PC = positive control deer.

However, 1 animal (cow 95) had a rectal temperature of 39.3°C on 4 dpi, a 0.7°C increase from the baseline mean of all animals in the study [38.6°C ± 0.4 (SD)]. Although there was variation between individuals in clinical pathological findings, no changes consistent with EHDV infection were observed during the study (data not shown). The mean total and differential white blood cell counts, haematocrit, platelet count, total protein, fibrinogen, PT, and APTT, were all generally within laboratory reference limits throughout the trial. At necropsy, no virus was isolated from spleen, lung, or lymph node of the 2 cows that did not have detectable viraemia on 18 dpi, but EHDV-7 was isolated from spleen and lymph node from the 1 cow that remained viraemic. The virus used in the inoculum was virulent to WTD, as the positive control had a detectable viraemia on 3 dpi (10^{3.63} TCID₅₀/ml). By 4 dpi, the animal began to exhibit clinical signs consistent with EHD in WTD (Howerth *et al.* 2001) including: decreased activity, lethargy, fever, erythema of non-haired skin, hyperaemia of mucous membranes, and subtle respiratory difficulty culminating in death on 5 dpi prior to sampling. At necropsy, this animal had a severe, pleural effusion (~ 1,200 ml). Post-mortem examination of cows was unremarkable.

Calf trial

Table II shows the results for virus isolation and titration from *C. sonorensis* that were membrane-fed an infectious blood meal and which were allowed 10

Table II. Blood feeding and virology results for *Culicoides sonorensis* responsible for transmission of serotype 7 of the Epizootic haemorrhagic disease virus (EHDV-7) to two calves in midge inoculation group. Midges were given an infectious blood meal (10^{6.27} TCID₅₀ EHDV-7/ml), held for 10 days at 26°C, allowed to feed on susceptible calves and processed immediately afterwards for virology.

Calf ID	No. midges feeding ^a	Percent positive ^b (no. positive/n)	Percent positive with high titer ^c (no. ≥ 10 ^{2.7} /no. pos.)	GMT of high titer midges ^d
84	136	33 (45/136)	84 (38/45)	10 ^{2.6} TCID ₅₀ ^e
89	150	33 (50/150)	78 (39/50)	10 ^{3.65} TCID ₅₀

^a Number of midges that took a blood meal from a naive calf 10 days after imbibing an infectious blood meal through a membrane.

^b Percent of midges positive by virus isolation immediately after feeding on naive calves.

^c High titer = titer of ≥ 10^{2.7} TCID₅₀/midge, considered potentially competent.

^d Geometric mean titer of midges with high titer (≥ 10^{2.7} TCID₅₀/midge).

^e Virus titer expressed as 50% tissue culture infective doses (TCID₅₀) per midge.

days later to feed on calves of inoculation group 3. Thirty-three percent of midges in both feeding groups (calf 84 and calf 89) were virus isolation positive. The percentage of these infected midges with a high virus titer (≥ 10^{2.7} TCID₅₀/midge) was high (78% and 84%, respectively). All calves had detectable viraemia 3-18 dpi (end of study); viraemia and serological profiles are presented in Table III. Peak viraemia occurred 7-10 dpi (10^{2.63}-10^{3.5} TCID₅₀/ml). Geometric mean of the peak blood virus titers were nearly identical between inoculation groups: group 1 (ID/SC), 10^{3.03} TCID₅₀/ml; group 2 (ID/SC/IV), 10^{2.8} TCID₅₀/ml; group 3 (midge), 10^{3.0} TCID₅₀/ml. Kinetics of viraemia profiles by inoculation group over the sampling period were similar (Figure 1). Precipitating antibodies were detected in all infected calves by 10 dpi, with 1 calf (calf 80) testing positive on 7 dpi. Peak neutralizing antibody titers (160 - ≥ 320) were detected on 18 dpi (end of study) (Table III). All calves behaved normally throughout the study and no clinical signs consistent with reports of EHD in cattle were observed. However, elevated rectal temperature was documented in both calves in inoculation group 2 (ID/SC/IV) on 1 dpi and again on 6-7 dpi. Rectal temperature for calf 77 was 39.9°C (1 dpi) and 40.2°C (7 dpi), an increase of 1.2°C and 1.5°C, respectively [calf 77 baseline mean rectal temperature, 38.7°C ± 0.1(SD)]. Rectal temperature for calf 93 was 40.4°C (1 dpi) and 39.6°C (6 dpi), an increase of 1.6°C and 0.8°C, respectively [calf 93 baseline mean rectal temperature, 38.8°C ± 0.1(SD)]. Baseline mean rectal temperature for each animal was determined by measuring rectal temperature daily for 5 days prior the beginning the study. No clinical pathological changes consistent with EHDV infection were observed during the study (data not shown). The mean total and differential white blood cell counts, platelet count, total protein, and fibrinogen were all generally within laboratory reference limits throughout the trial. Post-mortem

Table III. Comparison of viremia profiles and serologic responses of Holstein calves experimentally infected with serotype 7 of the Epizootic haemorrhagic disease virus (EHDV-7).

Animal ID	Inoc. route	Test	Day post-inoculation							
			0	1	3	5	7	10	13	18
71	ID/SC	VT ^a	–	ND ^c	<2.3	<2.3	2.63	2.63	<2.3	<2.3
		SNT ^b				<10	<10	10	80	160
80	ID/SC	VT	–	ND	<2.3	2.73	3.5	3.3	<2.3	<2.3
		SNT				<10	10	40	80	≥320
77	ID/IV/SC	VT	–	<2.3	<2.3	2.3	2.97	2.8	<2.3	<2.3
		SNT				<10	<10	20	160	≥320
93	ID/IV/SC	VT	–	<2.3	<2.3	<2.3	2.5	2.8	<2.3	<2.3
		SNT				<10	<10	40	80	160
84	Midge	VT	–	ND	<2.3	<2.3	<2.3	2.73	<2.3	<2.3
		SNT				<10	<10	10	40	160
89	Midge	VT	–	ND	<2.3	<2.3	2.73	3.3	<2.3	<2.3
		SNT				<10	<10	20	40	160
NC ^d	ID/IV/SC	VT	–	–	–	–	–	–	–	–
		SNT				<10	<10	<10	<10	<10

^a VT = virus titration, reported as log₁₀ median tissue culture infective doses per ml. Minimum detectable titer of 10^{2.3} TCID₅₀/ml.

^b SNT = serum neutralizing titer to EHDV-7, reported as reciprocal of the dilution.

^c ND = not done.

^d NC = negative control calf.

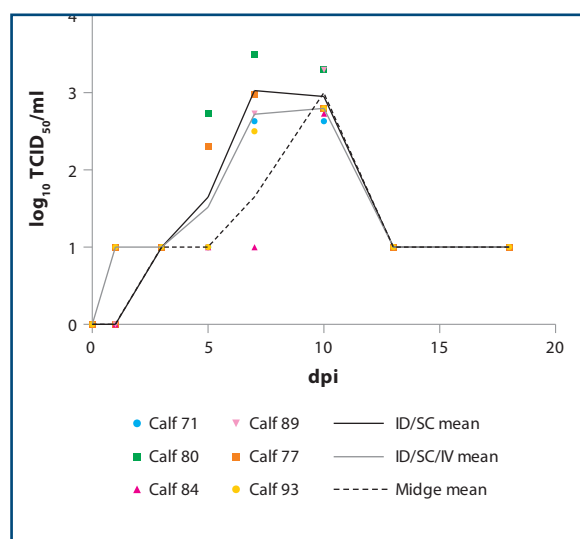


Figure 1. Inoculation group mean and individual virus titer of blood from calves experimentally infected with serotype 7 of the Epizootic haemorrhagic disease virus (EHDV-7). Group 1 (ID/SC): calves 71 and 80; group 2 (ID/SC/IV): calves 77 and 93; group 3 (midge): calves 84 and 89.

examination of the calves was unremarkable and EHDV-7 was isolated from 1 or more tissues from 5 of the 6 calves: spleen (calves 71, 77, 80, and 84), lymph node (71, 77, 84, and 89), and lung (calf 84). Tissues from calf 93 were virus isolation negative despite being viraemic on the day of necropsy (18 dpi).

A total of 1,380 *C. sonorensis* took a blood meal from 1 of the 4 calves with low-titer viraemia (< 10^{2.3}

TCID₅₀/ml) on 18 dpi: calf 77, n = 392; calf 89, n = 333; calf 80, n = 219; calf 84, n = 436. After a 10-day incubation at 26°C, 617 blood fed midges survived to be processed in pools of 5 for virus isolation: calf 77, n = 194; calf 89, n = 133; calf 80, n = 94; calf 84, n = 196. Thus, midge survivorship over the 10-day incubation period ranged from 40–49%. None of the 124 processed midge pools were positive by virus isolation after 2 passages.

Discussion

These 2 studies demonstrate the susceptibility of US Holstein cattle to infection with EHDV-7, a serotype exotic to North America. No overt clinical signs consistent with field reports of EHD in cattle were observed during infection of either cows or calves. However, the 2 calves in inoculation group 2 (ID/SC/IV) had transient elevations in rectal temperature. This is not surprising considering the artificial nature of this inoculation method. A similar response has been observed in calves infected with BTV-8 by combination IV/SC injection (Dal Pozzo *et al.* 2009).

Direct comparisons between the cow and calf trials should be made cautiously, as they were distinct trials using different virus preparations and cattle from herds located in 2 geographic regions. However, the observed variation in viraemia between the 2 trials is interesting. While all 4 cows seroconverted, only 3 of 4 had detectable viraemia by virus isolation. Viraemia was of low virus titer (< 10^{2.3} TCID₅₀/ml) and

while 1 cow remained viraemic for the duration of the study (18 dpi), viraemia in the other 2 cows was of short duration (8 days) (Table II). In the calf study, all 6 animals were virus isolation positive by 3 dpi and developed viraemia that peaked ($10^{2.63-10^{3.5}}$ TCID₅₀/ml) 7-10 dpi (Table III). All 6 calves remained viraemic ($<10^{2.3}$ TCID₅₀/ml) on 18 dpi (end of study). Peak viraemia in both calves infected by *Culicoides* bite (group 3) occurred on 10 dpi, whereas the peak was split between 7 and 10 dpi for calves in both groups 1 and 2. Although this may be an artifact of small sample size and sampling interval. If the observed differences are real, they would need to be elucidated through additional studies that include more animals and more frequent sample collection. Despite being subjective, the clearance of the low-titer viraemia in cows 10 and 25, and the drop in virus titer in all 6 calves, was temporally associated with increasing neutralizing antibody titers. Previous studies in cattle and WTD have shown a similar drop in virus titer, or virus clearance, coincident with seroconversion (Abdy *et al.* 1999, Quist *et al.* 1997b), although cytokine response may also be involved (Quist *et al.* 1997a, Sharma 2004). Another factor related to duration of viraemia is the peak virus titer (Quist *et al.* 1997b), which was low in the cows, thus the short duration viraemia observed was not surprising.

While duration of viraemia certainly varies, cattle and WTD can remain viraemic for up to 44 and 59 days, respectively (Abdy *et al.* 1999, Gaydos *et al.* 2002) because virions reside within pits on the surface of erythrocytes, potentially shielding them from co-circulating antibodies (Aradaib *et al.* 1997, Stallknecht *et al.* 1997). The epidemiological significance of low-titer viraemia in cattle and other ruminants infected with EHDV, such as those observed here, is not well-understood because the minimum blood virus titer infectious to the *Culicoides* vector is not known. However, such information is critical to understanding whether convalescent or sub-clinically infected ruminants are a source of EHDV for *Culicoides*. This is also important to understand in terms of potential virus overwintering mechanisms in certain ecological and climatic regions. In domestic sheep and cattle infected with BTV, virus was isolated from a small number of midge pools after feeding on viraemic animals 21 dpi (Bonneau *et al.* 2002). In our follow-up study in calves, we attempted to obtain some preliminary information regarding this question. All 124 pools (ca. 5 midges/pool) of *C. sonorensis* that fed on calves with low-titer viraemia were negative for EHDV-7 by virus isolation, indicating no *Culicoides* became infected. This is also supported by the fact that all 45 of the 0 dpf midges individually processed immediately after taking a blood meal also tested negative, indicating they

did not ingest an infectious blood meal. Although not conclusive, these data suggest this means of infection in *Culicoides* is inefficient. However, *Culicoides* attack rates can be very high in nature (Smith and Stallknecht 1996, Smith *et al.* 1996) and given the relative low number of *Culicoides* tested in our study, additional studies using a far greater number of midges should be performed.

Despite the numerous reports of EHD in cattle, experimental characterization of the disease is largely lacking, as nearly all experimental infections of cattle with EHDV have yielded subclinical infections, including North American EHDV-1 (Bowen 1987, Gibbs and Lawman 1977), EHDV-2 (Bowen 1987, Abdy *et al.* 1999, Aradaib *et al.* 1994), and EHDV-6 isolates (Ruder *et al.* 2015); Australian isolates of EHDV-5, EHDV-6, EHDV-7, and EHDV-8 (Uren 1986); a Reunion Island EHDV-6 isolate (Breard *et al.* 2013); Turkish and Moroccan isolates of EHDV-6 (Batten *et al.* 2011); and an Israeli isolate of EHDV-7 (Eschbaumer *et al.* 2012). The only study to produce disease was an experimental infection of Japanese cattle with EHDV-2 (strain Ibaraki) (Omori *et al.* 1969), which reproduced disease consistent with field cases in a subset (6/25, 24%) of calves. However, transient fever and leukopenia were more consistent and present in 96% (24/25) and 68% (19/25) of calves, respectively. The calves in that study were serially infected by IV inoculation with defibrinated blood from natural infections, whereas cows in our study were administered a blood inoculum prepared in WTD using SC and ID injection. Previous BTV studies (MacLachlan *et al.* 2008, Eschbaumer *et al.* 2010, Caporale *et al.* 2014) suggest that inoculation with naturally BTV infected tissues induces more severe disease in livestock compared with inoculation with cell culture-adapted virus. It has been suggested that this observation may be the result of decreased population diversity after virus passage in mammalian cell culture (Caporale *et al.* 2014). The experimental induction of EHD in cattle by Omori and colleagues (Omori *et al.* 1969) suggests this may also occur with EHDV in cattle. However, this remains unexplored with EHDV in cattle and inoculating cattle with field material was not possible in this study because of virus import regulations and the lack of infectious material banked during the outbreak in Israel. However, regardless of inoculation methods used, the disparate outcomes of EHDV-7 infection in WTD (where virulence is clearly demonstrated) and cattle suggest that the species of the mammalian host is a critical factor in determining clinical outcome. Also, whether initial passage of the virus through cattle instead of WTD would change the outcome of the cow study remains an open and intriguing question. Although, there is no evidence to suggest that EHD viruses circulating in sympatric WTD and cattle are distinct (Ananbalanga and Hause 2014).

As previously demonstrated with BTV in cattle, it is plausible that altering our inoculation methods may help to induce experimental EHD and subsequent research should explore this possibility further. However, it is important to note that a possible reason for so many experimental challenges yielding subclinical EHDV infections in cattle is the likelihood that this is the predominate outcome of infection in nature. During the reported EHD outbreaks in cattle, morbidity rates are frequently low, with overt disease reported in only a small portion of the herd. For instance, during EHDV-2 (strain Ibaraki) outbreaks in Japan, in-herd morbidity rates ranged from 0.01% to 12.6% despite widespread infection (Omori *et al.* 1969). Given the low number of experimental animals generally used, replicating a disease that affects so few individuals in nature is inherently difficult. Furthermore, additional factors that cannot be replicated experimentally are the adverse environmental conditions that may be present during EHD outbreaks. For instance, given the strict seasonality of EHD, weather conditions can be hot and dry and factors such as heat stress, drought, nutritional stress, and other adverse conditions that may impact individual animal susceptibility cannot be ethically simulated during animal research. Additionally, the role of co-infections with respiratory or immunosuppressive pathogens has not been explored in cattle. None of these factors are proven to play a role in the pathogenesis of EHD in cattle but sound diagnostic investigation of field outbreaks may help to elucidate potential predisposing factors, if present, which can then guide future experimental work.

One potentially important aspect of the EHDV-7 outbreak in Israel that may have not been fully replicated during our trial was the fact that the cattle population in Israel was immunologically naïve to EHDV. Although precipitating and neutralizing antibodies against EHDV were not detected in the cows procured for the study, the animals were mature milking cows (> 4 years) and EHDV transmission is common throughout much of Georgia (Stallknecht *et al.* 1995, Odiawa *et al.* 1985). While humoral immunity is important at the individual and herd level (Gaydos *et al.* 2002a), studies to understand other host defense mechanisms, such as cellular and non-specific antiviral responses to EHDV infection, are limited (Abdy *et al.* 1999, Quist *et al.* 1997a, Sharma. 2004). Although innate resistance to EHDV among WTD subspecies from endemic regions has been demonstrated (Gaydos *et al.* 2002b), it is not known whether a similar scenario functions in distinct cattle populations. Such a scenario, if it exists, may have further complicated our attempt to replicate clinical disease. The calves used in our second study were acquired from a closed herd in New York, a state from which EHDV has been

historically absent, in an attempt to better control for this possibility.

It is well-known that saliva from certain arthropod vectors can play a significant role in arbovirus transmission (Conway *et al.* 2014). Although this remains an understudied area in *Orbivirus* transmission, it has been shown that *Culicoides* saliva can modify EHDV and BTV particles (Darpel *et al.* 2009), as well as modulate the host immune response (Pages *et al.* 2014).

Culicoides-host interactions have not been investigated as these relate to EHDV infection. Aside from inoculum preparation in a *Culicoides* cell line (KC cells) (Batten *et al.* 2011), the *Culicoides* vector is an important variable that has been absent in all previous experimental infections of cattle with EHDV. In fact, to our knowledge, this study represents the first experimental transmission of EHDV to cattle by *Culicoides* bites. This lack of consideration for the insect vector in ruminant infection represents a significant research gap in itself. Future studies should aim to better understand the role of *Culicoides* spp. in EHDV infection and pathogenesis in the various ruminant hosts.

Despite this not being a comparative pathogenicity study, similar to a previous study with EHDV-6 (Ruder *et al.* 2015) marked variation in susceptibility to EHD between cattle and WTD was observed. The cows and positive control WTD received the same inoculum, yet the outcome of infection was drastically different, as the cows all had subclinical infection, but the WTD died peracutely from massive pleural effusion. Furthermore, considering other studies we have performed with this isolate of EHDV-7 (Ruder *et al.* 2012 a, b), we have experimentally infected a total of 10 WTD, and 8/10 either died or required euthanasia due to the severity of disease. This is in stark contrast to what we observed experimentally in cattle. The underlying reasons for the marked variation are not understood, although differences in susceptibility to virus-mediated endothelial cell injury, and the subsequent cellular response, between species may play a role, as such differences between cattle and sheep pulmonary endothelium have been demonstrated with BTV (DeMaula *et al.* 2001, DeMaula *et al.* 2002 a, b). It is also possible that cellular and non-cellular immune response to infection varies between WTD and cattle, although this is largely unexplored (Abdy 1999).

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