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Short communication

Cohabitation of pregnant white-tailed deer and cattle persistently infected with *Bovine viral diarrhea virus* results in persistently infected fawns

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ABSTRACT

Economic losses due to infection with *Bovine viral diarrhea virus* (BVDV) have prompted introduction of organized control programs. These programs primarily focus on the removal of persistently infected (PI) animals, the main source of BVDV transmission. Recently, persistent BVDV infection was demonstrated experimentally in white-tailed deer, the most abundant wild ruminant in North America. Contact of cattle and white-tailed deer may result in interspecific BVDV transmission and birth of persistently infected offspring that could be a threat to control programs. The objective of this study was to assess the potential for interspecific BVDV transmission from persistently infected cattle cohabitated with pregnant white-tailed deer. Seven female and one male white-tailed deer were captured and bred in captivity. At approximately 50 days of gestation, two cattle persistently infected with BVDV 1 were cohabitated with the deer. In a pen of approximately 0.8 ha, both species shared food and water sources for a period of 60 days. Transmission of BVDV as indicated by seroconversion was demonstrated in all exposed adult deer. Of the seven pregnancies, four resulted in offspring that were infected with BVDV. Persistent infection was demonstrated in three singlet fawns by immunohistochemistry and ELISA on skin samples, PCR, and virus isolation procedures. Furthermore, two stillborn fetuses were apparently persistently infected. This is the first report of BVDV transmission from cattle to white-tailed deer using a model of natural challenge. Under appropriate circumstances, BVDV may efficiently cross the species barrier to cause transplacental infection and persistently infected offspring in a wildlife species.

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1. Introduction

Bovine viral diarrhea virus (BVDV), a Pestivirus in the family *Flaviviridae*, is capable of infecting various mammalian host species in the order Artiodactyla (Vilcek and Nettleton, 2006). Cattle persistently infected (PI) with BVDV are considered the most important source of virus, however PI wildlife including white-tailed deer (*Odocoileus*

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virginianus) has been reported (Passler et al., 2007; Vilcek et al., 2000). Identification of heterologous PI hosts may have important implications for the epidemiology of BVDV, as these non-bovid PI animals may serve as reservoirs for BVDV. The livestock–wildlife interface is of great concern in the control of the related Pestivirus *Classical swine fever virus* (CSFV), where great measures are taken to prevent contact of domestic swine and wild boars, which are considered a reservoir of CSFV (Ruiz-Fons et al., 2008). Survival of PI wildlife to adulthood has been demonstrated in an eland and in surveys of hunter-harvested deer, and this may contribute to interspecific transmission (Passler et al., 2007; Vilcek et al., 2000). Reintroduction of BVDV into BVDV-free cattle herds by wildlife reservoirs has been speculated and certainly could present a serious threat to BVDV control efforts that are currently evolving in the United States (Sandvik, 2004; Simpson, 2002).

Experimental intranasal inoculation of a pregnant white-tailed deer with a BVDV 2 isolate resulted in the birth of a PI fawn and a mummified fetus (Passler et al., 2007). This PI fawn consistently shed high titers of BVDV, as indicated by repeated nasal swab virus isolations, thus demonstrating that PI white-tailed deer may be an efficient source of BVDV (Passler et al., 2007). The purpose of this study was to evaluate the potential for interspecific BVDV transmission using a natural model of transmission through cohabitation of PI cattle and in-contact pregnant white-tailed deer. White-tailed deer are the most abundant free-ranging ruminant in North America and home ranges commonly overlap with those of pastured cattle.

2. Materials and methods

2.1. Animals

The research described herein was performed under the approval of the Institutional Animal Care and Use Committee of Auburn University (2006-1108). In December 2006, seven female and one male free-ranging white-tailed deer were captured by cannon net, as previously described (Hawkins et al., 1968). All animals were determined to be free from BVDV and BVDV antibodies by whole blood virus isolation and serum virus neutralization, respectively. Following sample collections, captured deer were translocated to a pen at the Captive Deer Research Facility. The 0.8-ha deer pen contained a source of free-choice water and two wooden and covered livestock feeders. Daily, a commercially available 16% protein, high fiber ration at approximately 1.8 kg per deer and free choice grass hay was fed. Natural mating occurred in captivity with peak breeding season in Alabama centered around 20 January.

On 8 March 2007, when gestation length of deer was estimated to be approximately 50 days, two female cattle known to be PI with BVDV were cohabitated with captive deer. On the day of translocation, blood, nasal swab, and skin biopsies (ear notch) samples were obtained from cattle to reconfirm their status as PI animals. In addition, RT-nPCR products from both cattle were further characterized by sequence analysis and determined that both cattle were PI with BVDV 1b strains (designated AU526 and KY16). Nasal swab virus isolation titers at the beginning of cohabitation were 3.5×10^5 $CCID_{50}$ for both BVDV strains and serum virus isolation titers were 6.2×10^3 $CCID_{50}$/ml for BVDV KY16 and 3.5×10^4 $CCID_{50}$/ml for BVDV AU526.

During cohabitation, PI cattle and deer shared feed and water sources as may occur in a North American farm setting. Amount of contact between cattle and deer was also observed visually and by intermittent time-lapse videography. Captive deer were observed daily from a distance for clinical signs of BVDV infection. Following 60 days of cohabitation, PI cattle were removed from the captive deer pen. Upon the removal of cattle, nasal swab virus isolation titers were 1.1×10^5 $CCID_{50}$/ml for BVDV KY16 and 6.2×10^4 $CCID_{50}$/ml for BVDV AU526. Serum virus isolation titers were determined to be 6.2×10^3 $CCID_{50}$/ml for BVDV KY16 and 2.0×10^4 $CCID_{50}$/ml for BVDV AU526. Following the removal of PI cattle, pregnancies of deer were allowed to advance without interference.

Parturitions were expected to begin at the end of July 2007, when daily inspections for neonatal fawns were performed. Blood, skin biopsy, and nasal swab samples were collected for virus isolation, virus neutralization, RT-nPCR, and immunohistochemistry (IHC) procedures. Following sample acquisition, fawns remained at the captive deer pen for an additional 24 h to ensure colostral intake and then were translocated to an isolation room to be hand-raised.

Hand-raised fawns were fed a commercially available, multi-species milk replacer at 15% body weight, and hay and water were available ad libitum. Post-mortem examinations were performed on all fawns that died or at time of euthanasia. At necropsy, tissues (lymph nodes, spleen, and thymus) were collected from fawns that had been identified to harbor BVDV at birth and virus isolation and IHC procedures were performed. Approximately 5 months after parturition, all adult deer were euthanized. At time of euthanasia, serum was collected for virus isolation and virus neutralization procedures.

2.2. Maternity testing

A skin biopsy sample was collected from all adult and neonatal deer and stored at −80 °C until genetic analysis was performed to verify the maternity of fawns (Anderson et al., 2002; DeYoung et al., 2003). Information of maternity testing was used to assign fawn identification according to doe number and twin fawns were designated by assignment of letters A and B.

2.3. Virus isolation

Virus isolations were performed on sera, whole blood and nasal swab samples from fawns at birth; whole blood samples from adult deer at capture and euthanasia; and tissues (lymph nodes, spleen, and thymus) collected from fawns at post-mortem examination. Samples were assayed for BVDV by passage through MDBK cells, as has been described previously (Givens et al., 2003).

2.4. Skin biopsy immunohistochemistry

Immunohistochemical (IHC) detection of BVDV antigen was performed on formalin-fixed paraffin-embedded skin biopsies using a monoclonal antibody, 3.12F1 (Blas-Machado et al., 2004). Furthermore, at post-mortem examination, tissue samples (lymph node, spleen, and thymus) were collected from fawns that had been identified to harbor BVDV at birth and processed for IHC using the 3.12F1 monoclonal antibody. Antigen distribution in white-tailed deer, persistently infected with BVDV is analogous to that detected in PI cattle (Duncan et al., 2008; Passler et al., 2007).

2.5. Antigen capture ELISA

The BVDV antigen detection in skin biopsy samples was performed using a commercially available kit (IDEXX Laboratories, Westbrook, ME 04092, USA) developed for BVDV detection in bovine samples, according to the manufacturer's instructions. Presence or absence of BVDV within samples and classification as negative, suspect, or positive sample was established by using sample to positive (S/P) ratios of <0.20, 0.20–0.39, or >0.39, respectively.

2.6. Virus neutralization

A standard virus neutralization microtiter assay was used for the detection and quantification of antibodies in serum of adult deer at capture and euthanasia, and from those fawns that had not been determined to harbor BVDV at birth. Sera were tested for neutralizing antibodies to BVDV AU526 and KY16, as previously described (Givens et al., 2003). Antibody titer was defined as the inverse of the highest dilution with complete inhibition of staining by the immunoperoxidase test.

2.7. Reverse transcriptase polymerase chain reaction and sequencing

The BVDV was detected by a two-round rapid-cycle PCR assay on whole blood samples, serum, and tissues from fawns. This reverse transcription nested PCR (RT-nPCR) is characterized by an increased sensitivity when compared with conventional RT-PCR and has been previously described in detail (Givens et al., 2001).

RT-nPCR positive samples were purified using the QIAquick® PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's specifications and sequenced by automated dye terminator nucleotide sequencing using both the 5′ and 3′ primers (BVD 180 and HCV 368, respectively). Consensus sequences were determined for each sample using Align X® computer software (Vector NTI Suite 7.1, InforMax, Inc., Bethesda, MD, USA) and compared to nucleotide sequences of the challenge strains of BVDV.

3. Results

During cohabitation, both species were observed to favor a common area within the pen when resting, and

Table 1
Pertinent data on fawns

Fawn ID	Sex	Birth weight (kg)	Day of birth	Exposure[a]	Virus isolation				RT-nPCR		ELISA ear notch	IHC		Virus neutralization	
					Serum	WBC	Nasal swab	Tissues	Serum	Tissues		Ear notch	Tissues	AU526	KY16
17 A	M	ND	28 August 2007	32	–	+	–	ND	+	ND	ND	+	ND	ND	ND
18 A	M	1.22	31 July 2007[b]	62[b]	–	ND	–	+	ND	+	ND	+	+	ND	ND
18 B	F	1.34	31 July 2008[b]	62[b]	–	ND	–	+	ND	+	ND	+	+	ND	ND
19 A	M	2.65	30 July 2007	61	–	–	–	–	–	ND	–	–	ND	16	16
19 B	M	3.06	30 July 2007	61	–	–	–	ND	–	ND	–	–	ND	128	128
21 A	F	2.69	30 July 2007	61	–	–	–	ND	–	ND	–	–	ND	32,768	65,536
21 B	F	2.50	30 July 2007	61	–	–	–	ND	–	ND	–	–	ND	4096	131,072
23 A	M	2.40	21 August 2007	39	–	+	+	–	+	+	+	+	+	ND	ND
24 A	M	2.13	26 August 2007	34	–	–	–	ND	–	ND	–	–	ND	64	256
24 B	F	2.22	26 August 2007	34	–	–	–	–	–	ND	–	–	ND	128	256
25 A	F	3.03	4 August 2007	56	–	–	–	–	–	+	+	+	+	ND	ND

Key: ND—not determined.
[a] Gestational age at which exposure to PI cattle first occurred.
[b] Stillborn.

close interspecific contact appeared possible in this area. Time-lapse videography demonstrated that white-tailed deer and cattle did not feed at the same time. Rather, when feed troughs were filled, PI cattle ate first, followed by the deer within minutes thereafter. At observation from a distance, clinical signs of BVDV infection were not detected in the captive deer during or after exposure to PI cattle.

Nine live fawns (six twins and three singlets) and two twin stillborn fetuses were born within 30 days beginning on 30 July 2007. Pertinent data on fawns including results of virological assessment are presented in Table 1. All live fawns appeared healthy and vigorous when found. The fawns were determined to be full-term and body weights at birth were in the normal range (Haugen, 1959). The twin stillborn fetuses weighed below normal range and appeared to be pre-term according to dental eruption and appearance of hair-coat. Following a 24-h period at the captive deer pen to ensure colostrum intake, fawns were translocated to an isolation room and quickly adapted to bottle-feeding. By the end of August 2007 three of the eight hand-raised fawns (19A, 23A, and 25A) had died, prompting the decision to leave any fawns born thereafter with their dams. This decision was made to remove any artificial factors of hand-raising that may have been involved in fawn mortality. Only one fawn (17A) was born after this decision had been made.

Evidence of BVDV infection was detected in the three singlet fawns (17A, 23A, and 25A) and both stillborn fetuses (18A and 18B). All twin fawns were negative for BVDV infection according to virological assessment. From the samples collected on the first day of life, BVDV was isolated from the buffy coat of two fawns and the nasal swab sample of one fawn. Serum virus isolation was negative in all live-born fawns. In the stillborn fetuses, virus was isolated from four of five tissues, including lungs, spleen, thymus and lymph node. Ileal samples of these fetuses were microbiologically contaminated and BVDV was not isolated.

Diffuse antigen distribution was detected with IHC on ear notch samples in the three singlet fawns. The IHC staining pattern resembled those from PI cattle and a previously reported PI white-tailed deer (Passler et al., 2007). Ear notch samples from stillborn fetuses also were positive on IHC. In the skin samples from twin fawns, BVDV antigen was not detected. Tissue samples collected from singlet fawns and stillborn fetuses at post-mortem examination were positive for BVDV antigen, with multi-focal to diffuse distribution within tissue sections.

To substantiate results from IHC, ear notch biopsies from all fawns with the exception of the fawn that remained with its dam (17A) were processed for antigen detection by ELISA. Bovine viral diarrhea virus antigen was detected in two fawns, of which one fawn was classified as positive and the second as suspect, according to S/P ratio.

RT-nPCR was performed on sera of all live-born fawns at birth and demonstrated presence of viral RNA in the same two fawns that were positive on WBC virus isolation. At death, tissue samples were collected from fawns and positive RT-nPCR results were detected in the thymus and lymph nodes of one fawn, and spleen and thymus of a second fawn. In both stillborn fetuses, samples from lungs, spleen, lymph node, and thymus were positive on RT-nPCR. Genotyping of RT-nPCR products was performed by sequence analysis and demonstrated that the three singlet fawns were infected with BVDV strain AU526, while the stillborn fetuses were infected with BVDV strain KY16.

Virus neutralization was performed on serum samples from those fawns that did not harbor BVDV at birth and BVDV antibodies were detected in all samples. Antibody titers varied amongst fawns and were appreciably greater in a pair of twins (21A and 21B). Comparison of virus neutralization results from sera of adult deer at time of capture and time of euthanasia demonstrated seroconversion in all adult animals (Table 2). Virus was not isolated from adult deer at capture or time of euthanasia.

Table 2
Serum antibody titers of adult deer at capture and at euthanasia

	BVDV AU526		BVDV KY16	
	Capture	Euthanasia	Capture	Euthanasia
Buck	<1:4	1:128	<1:4	1:256
Doe 17	<1:4	1:1024	<1:4	1:32
Doe 18	<1:4	1:256	<1:4	1:256
Doe 19	<1:4	1:64	<1:4	1:16
Doe 21	<1:4	1:256	<1:4	1:256
Doe 23	<1:4	1:1024	<1:4	1:128
Doe 24	<1:4	1:256	<1:4	1:512
Doe 25	<1:4	1:512	<1:4	1:64

4. Discussion

This study demonstrates that cohabitation of naïve pregnant white-tailed deer with cattle PI with BVDV results in interspecific transmission of BVDV and birth of PI white-tailed deer. This is the first report of BVDV transmission from cattle to white-tailed deer using a model of natural challenge. Identification of seropositivity in all adult deer and birth of PI offspring in four of seven pregnancies emphasize that BVDV may efficiently cross the species barrier to cause PI offspring in a wildlife species. The results of the present and two previous studies indicate that both species of BVDV are capable of inducing persistent infection in white-tailed deer (Duncan et al., 2008; Passler et al., 2007).

Interestingly, all PI fawns in the present study were singlets, while non-PI fawns were twins. This observation may be the result of coincidence but raises the question of BVDV effects on gestation in polytocous species. In a previous study, a viable PI white-tailed deer fawn was found in the same birthing area as a mummified fetus indicating that outcome of transplacental BVDV infection may vary among twin deer fetuses (Passler et al., 2007). The pathogenesis of transplacental BVDV infection is incompletely understood. Fetal infection with Pestivirus may be by a stochastic mechanism and varying outcome of infection is a common finding among fetuses in twin pregnancies (Scherer et al., 2001; Swasdipan et al., 2002). In twin sheep and goat fetuses, different combinations of virological and clinical findings have been reported, including viropositive with vironegative fetuses; viable with stillborn or autolyzed fetuses; or stillborn with autolyzed fetuses (Loken and Bjerkas, 1991; Scherer et al.,

2001; Swasdipan et al., 2002). The causes for these findings are unknown but infection of twin fetuses at different times or different fetal responses to infection have been suggested (Scherer et al., 2001). Further research comparing the factors that are involved in the varying outcome of fetal BVDV infection in polytocous species may contribute valuable information to understanding the pathogenesis of transplacental viral infections.

In white-tailed deer, the gestational age of fetuses at which persistent infection may occur is unknown; however in a previous report a PI fawn resulted from intranasal inoculation of the doe on day 52 of pregnancy (Passler et al., 2007). A white-tailed deer that was experimentally inoculated at approximately 42–49 days of gestation also gave to birth to PI offspring (Duncan et al., 2008). In the present study, a cohabitation time of 60 days was chosen to allow for contact between deer and PI cattle at an estimated pregnancy stage of 40–100 days based on extrapolation of knowledge from cattle. In a recent study on vaccinal fetal protection in pregnant cattle, a cohabitation time of 98 days was chosen, resulting in transplacental infection of 14/14 bovine fetuses (Grooms et al., 2007). The amount of contact between PI cattle and white-tailed deer necessary to cause interspecific BVDV transmission is important epidemiologic knowledge that is needed to establish measures of biocontainment.

In cattle, BVDV commonly causes reproductive failure including infertility, embryonic resorption, fetal mummification, or abortion. The outcome of BVDV infection in pregnant animals is largely dependent on time of infection and infecting strain of BVDV (Grooms, 2004). Similarly, fetal resorption, mummification and abortions have been reported in white-tailed deer experimentally challenged with BVDV strains of bovine or cervine origin, respectively (Passler et al., 2007; Ridpath et al., 2007). All does in the present study carried their pregnancies to term and embryonic resorption or abortions were not observed. However, birth of two stillborn fetuses with low birth weights should be considered evidence of in-utero effects of BVDV. In contrast to PI fawns that were infected with BVDV AU526, the BVDV KY16 was isolated from both stillborn fetuses possibly indicating different strain-dependent effects on fetuses.

Transmission of BVDV in populations of white-tailed deer is likely influenced by human management of livestock and wildlife. Considering the pathogenesis of BVDV and the importance of PI hosts in the epidemiology of BVDV, winter-feeding may be an especially important factor of interspecific transmission of the virus. This study and a previous study indicate that a gestation length of approximately 50 days is suitable for induction of persistent infection in white-tailed deer, which coincides with the coldest months of the year when most intense winter feeding is likely practiced. A recent study performed in Minnesota reported seroprevalence rates of 41% and 25% in sampled white-tailed deer (Wolf et al., 2008). This is in contrast to a study performed in Alabama, where only 1.2% of animals were seropositive for BVDV (Passler et al., 2008). Longitudinal variations in management practices and climate may explain the different seroprevalence rates, as dependence of white-tailed deer on winter feeding is likely less important in more temperate climates.

Bovine viral diarrhea virus control programs are mainly focused on the removal of PI cattle from herds, thereby eradicating the most important source of BVDV infection for susceptible animals. With ongoing success of these programs, a main focus will have to be the prevention of BVDV reintroduction into BVDV-free herds. In conclusion, the present study emphasizes that BVDV is able to successfully cross species barriers and under appropriate circumstances may have the ability to establish wildlife reservoirs.

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