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Long-Term Clinicopathological Characteristics of Alpacas Naturally Infected with Bovine Viral Diarrhea Virus Type Ib

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Background: Substantial bovine viral diarrhea virus (BVDV)-related production losses in North American alpaca herds have been associated with BVDV type Ib infection.

Objectives: To classify and differentiate the long-term clinicopathological characteristics of BVDV type Ib infection of alpaca crias, after natural virus exposure. We hypothesized that persistently infected (PI) alpacas specifically demonstrate growth retardation, clinicopathological evidence of opportunistic infections, and early mortality.

Animals: Thirty-five crias naturally exposed to BVDV (18 acute, 3 chronic, 14 PIs), and 19 healthy cohort controls of 5 northeastern alpaca farms were prospectively evaluated over 2 years (September 2005–September 2008).

Methods: Observational cohort-control study.

Results: Chronically (viremia >3 weeks) and PI crias demonstrated significantly lower birth weights, decreased growth rates, anemia, and monocytosis compared with control animals. Common clinical problems of PI alpacas included chronic wasting, diarrhea, and respiratory disease. Median survival of PI alpacas that died was 177 days (interquartile range, 555) with a case fatality rate of 50% within 6 months of life. Transplacental infection was confirmed in 82% (9/11) of pregnant females on 1 farm, resulting in the birth of 7 PI crias (7/10 deliveries; 1 animal was aborted). Mean gestation at the beginning and end of BVDV exposure was 64 and 114 days, respectively.

Conclusions and Clinical Importance: Natural BVDV type 1b infection during early pregnancy resulted in a high incidence of PI offspring. Although PI alpacas may have distinct clinical characteristics, verification of persistent viremia in the absence of endogenous, neutralizing antibodies is essential to differentiate persistent from chronic infection.

Key words: Bovine viral diarrhea; Camelid; Persistent infection.

Bovine viral diarrhea virus (BVDV) has been recognized as a potential cause of serious illness, including diarrhea, reproductive loss, wastage, and death in South American camelids (SAC), posing a substantial threat to herd health.[1] BVDV is a member of the genus *Pestivirus* in the family Flaviviridae that exists in 2 biotypic forms (cytopathic and noncytopathic). Infection of both camelids[2] and cattle in early gestation with noncytopathic BVDV may produce persistently infected (PI) offspring that serve as a reservoir for viral spread as a consequence of lifelong shedding of BVDV from all mucosal surfaces.[3,4] Under experimental conditions, noncytopathic BVDV persistence may occur in 86–100% of calf fetuses infected in the susceptible gestational period.[5]

The incidence or risk of immune tolerance to BVDV and subsequent persistent viremia as a consequence of early fetal infection has not been previously explored in alpacas under conditions of natural virus exposure. In 2003, Wentz et al[6] failed to achieve PI in the offspring of 4 pregnant llamas experimentally inoculated with 3 llama-derived BVDV isolates (type 1a and 1b) during early gestation. BVDV type Ib isolates, however, recently were sequenced and identified in 35 crias classified as PI after natural infection in North America.[7] A BVDV seroprevalence study (May 2006 to July 2007) of 63 registered alpaca breeders, representing 26 US states, furthermore identified a 6.3% prevalence of PI crias at the time of investigation.[1] These findings underscore the importance of identification and elimination of PI in susceptible herds.

In cattle, BVDV has been associated with pathology in several organs, including the respiratory, hematologic, immunologic, neurologic, and reproductive systems. Acute infection of an immunocompetent animal most commonly leads to subclinical infection, although clinical signs of lethargy, fever, anorexia, decreased milk production, lymphopenia, thrombocytopenia, diarrhea, and death because of fulminating disease also may occur in some cases.[8,9] To date, only individual case reports have documented the clinical manifestation of BVDV infections in SACs. In llamas, BVDV infection has been associated with respiratory disease, abortion, ill thrift, and diarrhea.[10,11] Goyal et al[12] first isolated a noncytopathic BVDV type Ib of alpaca origin from a stillborn cria. Subsequently, the 1st PI alpaca cria was

Abbreviations:

BVDV	bovine viral diarrhea virus
IHC	immunohistochemistry
PCR	polymerase chain reaction
PI	persistent infection
SAC	South American camelids
SN	serum neutralizing antibody

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identified after evaluation of multiple abortions in eastern Ontario.[13] Postmortem evaluation further demonstrated a diagnosis of BVDV type Ib in 3 British alpacas with ill thrift and diarrhea.[11,14] Another PI case was identified after diagnostic evaluation of a premature cria with chronic respiratory disease, anemia, and failure to thrive.[15] Based on these individual reports, the clinical manifestations of BVDV in camelids appear diverse.

The insidious nature of BVDV may lead to substantial economic losses in the camelid industry if chronic or persistent virus carriers are not identified in the herd. The purpose of this study was to classify and differentiate the long-term clinicopathological characteristics of transiently infected and PI alpaca crias, after natural BVDV type Ib exposure. We hypothesized that PI alpacas may be differentiated from healthy controls and transiently BVDV-infected alpacas based on growth retardation, clinicopathological evidence of opportunistic infections, and early mortality.

Materials and Methods

Animals

Thirty-five alpaca crias and 1 fetus naturally exposed to BVDV type Ib at 5 different northeastern US alpaca farms were evaluated between September 2005 and September 2008. Animals were included in the study based on positive whole blood polymerase chain reaction (PCR) test results, virus isolation, BVDV type I serum neutralizing antibody (SN) analysis, and DNA sequencing at the Animal Health Diagnostic Center at Cornell University.

A diagnosis of persistent BVDV infection was based on a minimum of 2 consecutive positive whole blood PCR results at >3-week intervals and subsequent immunohistochemistry (IHC) confirmation at necropsy of all animals that died. Live PI animals at the time of study completion were required to demonstrate persistent viremia in the absence of endogenous serum neutralizing antibodies to BVDV. Chronic infection was differentiated from acute infection based on extended viremia (>3 weeks) followed by an increasing BVDV type I neutralizing antibody titer. Virus isolation confirmed positive whole blood PCR results. In total, data were collected from 14 persistently BVDV-infected crias, 1 aborted fetus, 18 animals acutely exposed to BVDV, 3 chronically infected (Fig 1), and 19 control animals consisting of non-BVDV-infected crias from the same cohort.

Chronically infected and PI crias were removed from their originating farms and raised in group isolation over a period of 2 years. In contrast, all acutely infected and control animals were raised in their home environment. Management and environmental conditions for control and BVDV-infected animals were similar, including access to fresh grass, free choice hay, and 0.5 kg concentrate[a] per adult animal per day, as directed by the owner. All animals >4 months of age were dewormed with Doramectin[b] every 6 weeks.

Data Collection

Signalment, history, clinical signs, sequential PCR and SN, complete necropsy, and IHC were obtained whenever possible, and outcome was documented based on survival, euthanasia, or death. Clinical examinations of acutely infected animals were limited to owner observation. In contrast, the behavior, clinical examination abnormalities, feed intake, nursing activity, and fecal output were monitored daily by a licensed veterinarian for chronically infected and PI animals. Body weight was recorded at monthly intervals.

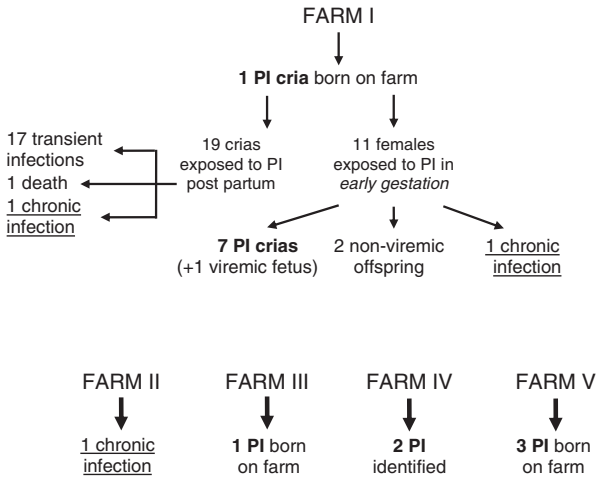

Fig 1. Origin of persistently and chronically bovine viral diarrhea virus-infected alpaca crias. Cria I, PI (*farm I*—born 2005); Cria II, chronically infected (*farm I*—born 2005); Cria III, chronically infected (*farm II*—born 2006); Cria IV, PI (*farm III*—born 2006); Cria V–VII, PI (*farm V*—born 2006); Cria VIII–XIV, PI (*farm I*—born 2006); Cria XV, chronically infected (*farm I*—born 2006).

CBC analyses were performed at the time of study enrollment and biannually thereafter, as dictated by the duration of the crias' survival. Hematological results obtained at sequential time points were averaged for each animal in order to limit bias because of age and repeated analysis of long-term survivors. A single CBC dataset per animal therefore was compared between age-matched BVDV infected (mean age, 7.9 months) and cohort control animals (mean age, 7.6 months). A CBC also was obtained within 3–6 weeks after resolution of viremia in chronically infected animals.

At postmortem examination, tissue samples were collected for histopathology, fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin according to standard histologic technique. Five-micron-thick sections were prepared and stained routinely with hematoxylin and eosin for microscopic examination. Sections also were stained for immunohistochemical detection of BVDV antigen by use of an avidin-biotin alkaline phosphatase method.[16]

Virus Isolation

All virus isolations were performed using primary bovine testicular cell cultures in T-25 flasks. Eagle's Minimal Essential Medium[c] was supplemented with 10% γ-irradiated fetal bovine serum free of antibodies to type 1 and type 2 BVDV. Samples for inoculation consisted of serum or mononuclear cells. Mononuclear cells were added to cell monolayers without freezing. After overnight incubation, the monolayers were rinsed with medium and MEM + 10% FBS was added. After 5–7 days, monolayers were trypsinized and a sample of the cells was tested for the presence of BVDV using a BVDV monoclonal antibody 20.10.6 in an indirect fluorescent antibody test. Negative cultures were retested after an additional 5–7 days of incubation.[7]

Serum Virus Neutralization (SN) Test

Serum was harvested from blood samples collected by direct venipuncture. Neutralizing antibody titers were determined by combining serial dilutions (1 : 4–1 : 512) of heat-inactivated (56°C for 30 minutes) serum with BVDV type 1 (Singer strain) or BVDV type 2 (strain 125; 100–300 $TCID_{50}$/50 μL) in 96-well microtiter plates for 1 hour at 37°C with 5% CO_2. After incubation, 20,000–

25,000 bovine testicular cells were added to each well and incubated 5 days at 37°C with 5% CO_2. Cells were examined microscopically for changes associated with viral cytopathic effects (CPE), and titers of virus-neutralizing antibodies were recorded as the reciprocal of the highest serum dilution that inhibited CPE, as described previously.[17]

Real-Time RT-PCR

A real-time RT-PCR assay (RRT-PCR) with a commercial real-time PCR unit[d] was utilized as described previously.[7] 5′ UTR RRT-PCR was performed with a commercial real-time PCR reagent kit.[e] The RRT-PCR mixture consisted of 12.5 μL of 2× Master Mix, 0.625 μL of 40× MultiScribe and RNase Inhibitor Mix, 0.3 μM of each primer, 5 μL of template RNA, 0.1 μM of BVDV probe, and water up to 25 μL. Thermocycling was performed at 48°C for 30 minutes to synthesize the first-strand cDNA, 95°C for 10 minutes to inactivate reverse transcriptase, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. A standard curve was constructed in each experiment using serial dilutions of the cell culture supernatant of the Singer strain of BVDV with a titer of 3×10^6 50% tissue culture infectious dose per milliliter ($TCID_{50}$/mL). The relative number of BVDV genome copies in the clinical samples was measured by comparing their threshold cycle (C_T) values to those of the standards. A threshold value usually is set when the fluorescence reaches 10 times the standard deviation of the baseline signal.

Statistical Methods

All historical, clinical, laboratory, and necropsy data were reported descriptively. Statistical differences between animal groups were evaluated via the independent samples *t*-test by commercial software,[f] with an accepted significance level of $P < .05$. All data were presented as mean ± standard deviation (SD) or median ± interquartile range based on the normality of data distribution (Kolmogorov-Smirnov test). The duration of BVDV infection and presence of serum neutralizing antibody responses were documented from the time of BVDV diagnosis to the time of death or completion of the study in September 2008 in surviving alpacas.

Results

Alpaca Farm I

A BVD PI alpaca cria (cria I) was born on *farm I* in September 2005. Its dam was exposed to BVDV type Ib before entering *farm I* at 168 days gestation, based on retroactive BVDV testing of the originating farm where 2 PI crias were identified. PI of cria I was confirmed by 3 consecutive positive whole blood PCRs between 6 and 20 weeks of age, virus isolation, and subsequent IHC at necropsy (4.7 months of age). At the time of parturition, the cria's dam was whole blood PCR negative and SN antibody positive (titer of 64). *Farm I* had no history of previous BVDV exposure and 72% of alpacas in direct contact (5 acre pasture) with cria I, seroconverted over the next 9 weeks (36/58 animals; 62%) or 14 weeks (6/58; 10%), respectively.

A total of 11 pregnant females in the 1st trimester of pregnancy were naturally exposed to BVDV over a period of 50 days (range, 11–87 days) based on seroconversion. The mean gestation of pregnant dams at the beginning of BVDV exposure was 64 days (95% CI: 47–82 days; range, 11–102 days). The virus was expected to circulate for a minimum of 2 weeks after removal of the PI and 1 chronically infected cria from the premises, extending average virus exposure until day 114 of pregnancy (95% CI: 100–130 days; range, 74–152 days). One exposed pregnant female was intentionally aborted at 146 days of gestation. BVDV infection of the aborted fetus was subsequently confirmed by IHC at necropsy. The remaining 10 exposed pregnant females were removed from the premises in the last trimester of pregnancy and allowed to deliver their crias at a private isolation facility (see "cohort study" below).

In addition, 19 juvenile, nonweaned alpacas were first exposed to BVDV at a mean age of 76 days (95% CI: 56–96 days; range, 1–121 days) on *farm I*, based on seroconversion and PCR analysis. Of these, 6 alpaca crias were viremic at the time of 1st PCR analysis and cleared the virus from their blood stream within 3 weeks of identification. Eighty-nine percent (17/19) of transiently BVDV exposed crias remained clinically healthy based on owner observation. Morbidity and mortality was limited to 2 viremic crias that were born 1 day before and 14 days after the birth of the PI cria in the same pasture. One neonate died peracutely, showing histologic evidence of pleural and subpleural hemorrhage, multifocal mucosal petechiae of the esophagus and urinary bladder, mild hepatocellular necrosis, diffuse vascular congestion, as well as villus and crypt epithelial cell necrosis within the small intestine. The second cria (cria II) was admitted to the Tufts Cummings School of Veterinary Medicine at 11 days of age, for an acute onset of yellow, watery diarrhea, and weakness progressing to collapse. Presenting clinical abnormalities included fever, tachycardia, weakness and recumbency, moderate lethargy, diarrhea, a grade II/VI systolic heart murmur, hyperemic mucous membranes, and hypovolemia. Cria II remained hospitalized for 21 days with a diagnosis of enterocolitis, confirmed sepsis and prolonged BVDV infection. Extended viremia was identified, based on 3 sequential whole blood PCRs at 12, 20, and 57 days of age, followed by confirmatory virus isolation.

Alpaca Farm II

Farm II identified 1 congenitally infected alpaca cria (cria III) with extended viremia, based on routine herd screening. Three whole blood PCR tests between 1 and 67 days of age confirmed prolonged viremia, in conjunction with increasing BVDV type 1 antibody titers.

Alpaca Farms III–V

Farm III detected 1 PI alpaca cria (cria IV) on routine herd screening, after the day of parturition. *Farm IV* similarly identified 2 PI alpacas (1 male, 1 female) at 7 and 19 months of age based on 2 consecutively positive whole blood PCRs, and confirmed the diagnosis by IHC at subsequent necropsy. In addition, *Farm V* detected 3 PI alpaca crias (crias V–VII) after parturition, obtaining sequentially positive whole blood PCR results, virus isolation and subsequent IHC confirmation at necropsy (5, 6, and 7.5 months of age).

Cohort Evaluation

Ten pregnant female alpacas from *farm I* with confirmed BVDV type Ib exposure during the 1st trimester of pregnancy (64–114 days of gestation) were removed from their originating premises and clinically studied throughout their last 60 days of gestation (summer 2006) at a private isolation facility. Clinical signs of illness were not observed throughout the adult animals' stay and 9 of 10 females delivered unassisted (stage 2 labor < 15 minutes). One alpaca, with a history of previous dystocia, had a breech delivery of a BVDV negative full-term dead fetus. In total, transplacental fetal infection was confirmed in 9/11(82%) pregnant female alpacas naturally exposed to BVDV during early gestation at *farm I*. This resulted in the birth of 7 PI alpaca crias (7/10 [70%] deliveries; 1 animal was aborted), 2 noninfected neonates (including the stillborn dystocia), as well as 1 congenitally infected cria (cria XV) with extended viremia. Prolonged viremia was identified, based on 6 consecutive BVDV whole blood PCRs at 4–251 days of age, confirmed by virus isolation.

Prospective Assessment of PI and Chronically Infected Crias

The above-described 8 intrauterine BVDV infected, live-born crias from *farm I* (7 PI, 1 chronically infected; VIII–XV) were raised in group isolation and prospectively evaluated over the next 2 years (2006–2008). Similarly, crias I–VII were transferred from their home farms and raised at an isolated facility, after confirmation of extended viremia (>3 weeks). In total, 12 live PI crias (7 female, 5 male) were enrolled for prospective assessment. Data collection also included clinicopathological information obtained retrospectively from 2 PI crias (1 male, 1 female) raised on *farm IV*.

PI crias were differentiated from 3 chronically infected crias (II, III, and XV; 2 female, 1 male) with prolonged viremia for >57 days, >67 days, and >251 days, respectively. A single positive SN titer of 4,096 was obtained for cria II at 129 days postpartum. Cria III was negative for serum neutralizing antibodies at 40 days postpartum and subsequently had titers of 32 (days 166 and 213), 768 (day 456), and 384 (day 529). Cria XV demonstrated sequential SN titers of 124 (day 0, postcolostrum), 64 (day 59), 48 (day 203), 96 (day 250), 384 (day 493), and 192 (day 566). All chronically infected crias were removed from PI exposure at 135 days (cria II), 486 days (cria III), and 523 days postpartum (cria XV), respectively, leading to a documented reduction in SN titers in the latter 2 animals.

Two noninfected females and 1 male cria born in isolation to 3 of the 11 BVDV-exposed pregnant dams from *farm I* (see "cohort evaluation" above) served as control animals. In the 2 years preceding and following PI births (2006), 16 healthy crias (10 female, 6 male) were born to the same 11 dams from *farm I* and were available as additional controls (half-siblings; years of parturition: 2004–2005 and 2007–2008).

Chronically infected (n = 3; $P = .031$) and PI crias (n = 11; $P < .001$) for which data were available showed significantly lower mean birth weights (6.6 ± 0.82 and 6.4 ± 1.0 kg, respectively) compared with noninfected, cohort control animals (n = 16; 8 ± 0.95 kg). A relative reduction in growth rates similarly was evident between control and PI crias over a 2-year observational period, during which body weights were significantly higher in controls at all time points (Fig 2). In contrast, gestational age was comparable between animal groups (PI: 332 ± 14 days, n = 11; chronically infected crias: 330 ± 5.7 days, n = 3; cohort controls: 334 ± 9 days, n = 19).

PI alpacas had significant anemia ($P = .001$) as well as relative ($P = .024$) and absolute blood monocytosis ($P = .038$) compared with control animals (Table 1). Serum

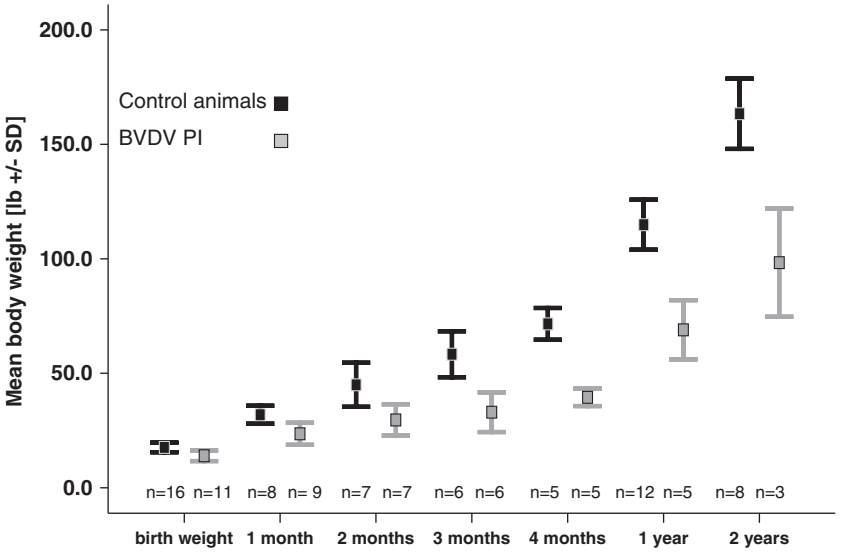

Fig 2. Growth curves of persistently bovine viral diarrhea virus-infected crias compared with healthy controls. BVDV PI, alpacas persistently infected with bovine viral diarrhea virus type 1b.

Table 1. Hematologic parameters of PI, chronically infected, and healthy control crias (mean ± SD).

Mean (SD)	Persistent Infection	Chronic Infection (PCR positive)	Chronic Infection (PCR negative)	Healthy Controls
n	10	3	3	16
WBC ($10^3/\mu L$)	8.1 (2.5)[a]	7.3 (2.1)[a]	12.0 (0.8)[b]	10.8 (4.4)[a,b]
Hct (%)	23.4 (3.7)[a]	26.1 (2.6)[a/b]	29.0 (5.7)[a/b]	28.9 (3.3)[b]
Neutrophils (%)	60.1 (18)[a]	56.3 (6.7)[a]	49.0 (8.3)[a]	52.7 (13.4)[a]
Lymphocytes (%)	31.9 (16.6)[a]	35.7 (5.8)[a]	45.0 (10.3)[a]	41.8 (16.2)[a]
Monocytes (%)	5.7 (3.6)[a/c]	6.7 (1.5)[a]	2.7 (0.6)[b/c]	2.1 (1.6)[b]
Neutrophils ($10^3/\mu L$)	5.1 (3.1)[a]	4.0 (1.8)[a]	6.1 (0.8)[a]	5.5 (2.3)[a]
Lymphocytes ($10^3/\mu L$)	2.4 (1.3)[a]	2.3 (1.0)[a]	5.2 (1.1)[b]	4.6 (3.4)[a/b]
Monocytes ($10^3/\mu L$)	0.41 (0.26)[a]	0.42 (0.18)[a]	0.3 (0.1)[a/b]	0.21 (0.14)[b]

Chronic infection, crias with extended BVD viremia >3 weeks; n, number of animals for which data were available (results of sequential analyses in individual animals were averaged before data entry); WBC, white blood cell count.

Changes in superscript letters within rows indicate significant differences between groups ($P < .05$).

iron concentrations were determined in 4 PI (mean, 47.7 ± 18.5 μg/dL; reference range for llamas, 70–148 μg/dL)[18] and 2 chronically infected alpacas (mean, 120 ± 17.4 μg/dL) at 2 separate time points, indicating low serum iron concentrations in all tested PI animals. CBC were indistinguishable between PI and chronically infected, viremic crias ($P > .05$). After virus clearance, however, recovered alpacas demonstrated a significant increase (normalization) in total WBC count ($P = .02$) and absolute lymphocyte counts ($P = .025$), as well as a relative reduction in monocytes ($P = .013$) compared with results obtained during viremia.

Thirteen of 14 PI alpacas (93%) suffered from various clinical illnesses during their lifetime, most commonly chronic wasting (9/14), diarrhea (7/14; recurrent in 4 animals), and respiratory tract disease (7/14, chronic intermittent signs in 3/7), which was associated with purulent, bilateral nasal discharge in 6 of 7 affected animals. In addition, transient cutaneous papillomas were present in 3 neonatal alpacas originating from the same farm. Periocular and mucocutaneous hyperkeratosis surrounding the lips (1), a congenital mature cataract (1), neonatal encephalopathy (1), septic arthritis (1), and suri-type fleece (1) were observed in 1 animal each. Unilateral corneal ulcers developed in 2 alpacas and were recurrent in 1 animal. As of December 2010, 2 PI alpacas have remained free of clinical signs for a period of 52 and 54 months, respectively, aside from 1 episode of transient diarrhea in 1 animal and evidence of cutaneous hyperkeratosis in both animals.

A diagnosis of partial or complete failure of passive transfer (FPT) was based on immunoglobulin G concentrations <800 mg/dL and was documented in 6/11 PI (55%), 2/3 chronically infected, and 0/13 cohort control animals for which data were available. Commercial plasma[g] (30 mL/kg) was administered to all animals with FPT. Of the 2 PI alpacas that remained clinically healthy long term, only 1 experienced FPT postpartum. Postcolostral serum neutralizing BVDV antibodies were detected in 4 of 9 PI crias for which data were available. One of these PI crias maintained a low seropositive BVDV titer (1 : 8) until the last sampling date before its death, at 123 days postpartum.

Overall, 50% of PI crias died acutely within 6 months after parturition (7/14), with a median age of 177 days (interquartile range, 555 days) at the time of death for all PI animals. The mortality rate reached 64% (9/14) and 79% (11/14) by 1 and 2 years, respectively. In contrast, all chronically infected alpacas and controls were alive at the end of the 2-year observation period. Detailed histopathological data, including analysis of viral distribution in PI crias that died are reported elsewhere (Henningson JN, Steffen DJ, Topliff CL et al. Systemic distribution of viral antigen in alpacas persistently infected with bovine pestivirus. Vet Path, under review).

Discussion

The current study allowed for a unique long-term, prospective characterization of transient (acute or chronic) and persistent BVDV infection in domestic alpacas because of natural noncytopathic BVDV type Ib exposure. Acute infection remained subclinical in most study alpacas, based on owner assessment. However, similar to our observations in 2 neonatal crias, transient infection may result in clinical signs of diarrhea, lethargy, oculonasal discharge, anorexia, and pyrexia in some animals.[2] Ecchymotic hemorrhages with hepatocellular and epithelial crypt cell necrosis of the small intestine were observed after the peracute death of 1 transiently infected alpaca cria. Systemic hemorrhage was most likely related to endotoxemia and suspected disseminated intravascular coagulation. Peracute BVDV infections also have been reported in cattle and may result in severe disease manifestation and higher fatality rates. In both cattle and calves, a thrombocytopenic, hemorrhagic syndrome has been associated with bloody diarrhea, ecchymoses, epistaxis, and petechiae. This syndrome has been experimentally linked to BVDV type 2 infection in cattle,[19] but has not been reported in camelids to date.

The current study is the 1st documentation of prolonged BVDV viremia in alpacas, which extended for more than 57, 67, and 251 days in 3 alpacas, respectively, after perinatal (n = 1) as well as intrauterine (congenital) BVDV infection (n = 2). The virus can be recovered from blood and nasal secretions of acutely infected cattle for 6–8 days, although higher virulence isolates may result in a longer duration of viral shedding.[16,20] PI animals are unable to produce serum neutralizing antibodies against the infecting noncytopathic BVDV strain because of the

presence of immune tolerance. PI therefore is routinely determined by the isolation of BVDV from peripheral blood leukocytes or serum on 2 separate occasions at least 3 weeks apart,[21] in the absence of endogenous, homologous neutralizing antibodies. However, PI animals can respond immunologically to heterologous strains of BVDV, so that a seropositive status cannot be utilized diagnostically to rule out PI.[22] Because ingestion of colostrum also may lead to measurable BVDV serum antibodies, a definitive differentiation between persistent and chronic infection in crias may only be possible once maternal antibodies have dissipated. Calves with maternal antibodies to BVDV type 1 and 2 are estimated to become seronegative by 141 days and 114 days postpartum, respectively.[23] In the current study, 1 PI alpaca cria still had a low (titer of 8) maternal BVDV antibody titer at 123 days after parturition. Serum antibody titers also remained low for the first 6 months of life in transiently infected crias with extended viremia, suggesting a delayed acquired immunity to BVDV and response to exogenous BVDV exposure because of cohousing with PI alpacas. One of the affected animals (cria III) became PCR negative before development of substantial serum neutralizing antibodies, which may suggest that both colostral and cell-mediated immunity play a role in BVDV elimination in the postnatal period. Protective immune responses against BVDV that may not be reflected by serum antibody titers can be mounted in calves by passive immunity.[24]

Transplacental infection was identified in 9/11 (82%) naïve, pregnant alpacas naturally exposed to BVDV type Ib during early gestation, with confirmed viral persistence in 7/10 live-born crias. Similarly, BVDV persistence has been reported in 86–100% of calf fetuses infected before reaching immunocompetence.[25] PI infection is the result of in utero, noncytopathic BVDV infection during the fetal development period between 45 and 125 days of gestation in cattle. This gestational period encompasses the time from the end of the embryonic stage to the development of fetal immunocompetence,[2] which has not been determined in camelids to date. The mean gestation of alpacas at the beginning and end of virus exposure was 64 and 114 days, respectively, in the current study. In comparison, a previous report documented the birth of a PI cria after BVDV type 1b exposure at 65 days of gestation.[13] Experimental infection with llama-derived BVDV isolates (type 1a and 1b) in 4 pregnant llamas between days 65 and 105 of gestation did not result in fetal infection or birth of PI crias in a previous study.[6] In contrast, PI crias were born to pregnant alpacas after intranasal inoculation with BVDV type 1b strains of alpaca or cattle origin in a recent report, whereas BVDV type 2 exposure did not produce persistent fetal infection despite ability to induce seroconversion.[26,27] These data support that unique BVDV type 1b genotypes are able to establish transplacental infection in alpacas after both natural and experimental viral exposure.[2] Transplacental infection therefore may maintain unique bovine BVDV 1b genotypes within the alpaca population whereas de novo introductions of BVDV genotypes appear to be low, based on previous reports.[7]

PI alpaca crias experience a high degree of morbidity and mortality, associated with several different clinical disease manifestations. These findings may relate to an impaired immune response in PI animals, facilitating opportunistic infection and early death. Reported clinical abnormalities of PI alpacas include stillbirth, low-grade pyrexia, atypical fleece, lethargy, unthriftiness, inappetence, low birth weight, poor weight gain, diarrhea, joint swelling, as well as signs of opportunistic dental, intestinal, upper or lower respiratory disease, based on individual case reports.[10,11,13–15] Similar observations were obtained from the current study, where low birth weights, chronic wasting, diarrhea, and respiratory tract disease were most prevalent in PI alpacas. Transiently infected alpacas with extended viremia also demonstrated low birth weights. Stunted growth was observed in 1 of 3 chronically infected animals, which also experienced prolonged postpartum morbidity because of enterocolitis and confirmed sepsis. These data support that the peripartum phenotypic characteristics of PI crias may be clinically indistinguishable from those of chronically infected crias with extended viremia.

There are several limitations of the current study, which included a heterogenous animal population from different farms. Certain observations, including postpartum behavioral analyses and monitoring, therefore were performed by the owners and may have limited our ability to identify subtle clinical changes in these animals. Management practices were similar for all animals, although some differences in environment and handling may have impacted our comparison between study animals and controls. The impact of genetic factors was minimized by choosing age-matched animals of similar genetic background (same dam) as control animals.

BVDV has been shown to replicate in bovine lymphocytes and macrophages, inducing lymphocyte depletion and sometimes neutropenia.[28] A recent study comparing BVDV PI and healthy heifers under field conditions identified a leukopenia and neutropenia, with relative lymphocytosis in PI versus control heifers. Blood monocytes were significantly decreased both in number and in proportion when compared with control heifers, suggesting that these cells are one of the major virus targets in cattle.[28] Acute, experimental challenge of postpartum calves with BVDV type 2 similarly induced a significant drop in all circulating leukocytes (neutrophils, lymphocytes, and monocytes) by days 3 or 5 postexposure.[29] In contrast to cattle, PI and chronically infected, viremic alpacas displayed both relative and absolute monocytosis compared with controls, which was likely a response to opportunistic infection. Additionally, significant anemia (Table 1) was observed in alpacas with persistent viremia. Low hemoglobin concentration and hematocrit similarly were documented in a PI alpaca and BVDV-infected llama of 2 previous reports.[10,15]

Hematologic parameters were indistinguishable between PI and chronically infected, viremic alpacas and may not aid in clinical differentiation between these patient groups. Nonetheless, chronically infected alpacas showed a significant increase (normalization) in both total WBC and absolute lymphocytes after resolution of viremia. However, total WBC, lymphocyte, and neutrophil counts were not statistically different between PI

alpacas and healthy controls. Previously, a report of a BVDV-infected llama identified leukocytosis, despite evidence of lymphoid depletion and thymic atrophy.[10] Our results suggest that persistent BVDV infection of alpacas under field conditions may have a minor or variable impact on lymphocytes and neutrophils, in comparison with results obtained after experimental, acute infection[29] or natural PI[28] in cattle.

Inadequate transfer of passive immunity (FPT) was prevalent in both PI (6/11, 55%) and chronically BVD-infected crias (2/3, 66%) and was most likely related to less vigorous or delayed nursing activity in viremic crias. In contrast, healthy half siblings (n = 16) born within the 2 years preceding and after a PI birth by the same dam showed adequate transfer of maternal immunoglobulins. Inadequate passive immunity therefore may contribute to opportunistic infection in PI alpacas within the first weeks of life. Opportunistic infection and impaired immune responses may contribute to early death in PI alpacas, because BVDV also is known to infect cells that are instrumental in the control of both the innate and acquired immune system.

The highest mortality (7/14, 50%) was observed within the first 6 month of life in PI crias of the current study. However, some PI alpacas were born without clinical abnormalities and were impossible to distinguish phenotypically form healthy cohorts, similar to observations in cattle.[2] Although stunted growth was common in PI crias, 2 animals of the current report grew normally and showed minimal morbidity long term. This observation further underscores the importance of routine BVDV-specific diagnostic testing (eg, PCR, serum neutralizing antibody testing, virus isolation) in order to identify PI and chronically infected camelids, which serve as a major source of viral spread within and among farms.

Footnotes

[a] Evans Alpaca Maintenance, Blue Seal Feeds, Londonderry, NH
[b] Dectomax, Pfizer Animal Health, Exton, PA
[c] Invitrogen/GIBCO, Grand Island, NY
[d] AB 7,900, Applied BioSystems, Carlsbad, CA
[e] Taqman EZ RT-PCR kit, Applied BioSystems
[f] SPSS, version 12, SPSS Inc, Chicago, IL
[g] Triple J Farms, Kent Labs, Bellingham, WA

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References

1. Topliff CL, Smith DR, Clowser SL, et al. Prevalence of bovine viral diarrhea virus infections in alpacas in the United States. J Am Vet Med Assoc 2009;234:519–529.

2. Walz PH, Grooms DL, Passler T, et al. Control of bovine viral diarrhea virus in ruminants. J Vet Intern Med 2010;24:476–486.

3. Coria MF, McClurkin AW. Specific immune tolerance in an apparently healthy bull persistently infected with bovine viral diarrhea virus. J Am Vet Med Assoc 1978;172:449–451.

4. Brownlie J, Clarke MC, Howard CJ. Experimental production of fatal mucosal disease in cattle. Vet Rec 1984;114:535–536.

5. Grooms DL. Reproductive consequences of infection with bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract 2004;20:5–19.

6. Wentz PA, Belknap EB, Brock KV, et al. Evaluation of bovine viral diarrhea virus in New World camelids. J Am Vet Med Assoc 2003;223:223–228.

7. Kim SG, Anderson RR, Yu JZ, et al. Genotyping and phylogenetic analysis of bovine viral diarrhea virus isolates from BVDV infected alpacas in North America. Vet Microbiol 2009;136:209–216.

8. Brock KV. The many faces of bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract 2004;20:1–3.

9. Campbell JR. Effect of bovine viral diarrhea in the feedlot. Vet Clin North Am Food Anim Pract 2004;20:39–50.

10. Belknap EB, Collins JK, Larsen RS, et al. Bovine viral diarrhea virus in New World camelids. J Vet Diagn Invest 2000;12:568–570.

11. Foster AP, Houlihan MG, Holmes JP, et al. Bovine viral diarrhoea virus infection of alpacas (*Vicugna pacos*) in the UK. Vet Rec 2007;161:94–99.

12. Goyal SM, Bouljihad M, Haugerud S, et al. Isolation of bovine viral diarrhea virus from an alpaca. J Vet Diagn Invest 2002;14:523–525.

13. Carman S, Carr N, DeLay J, et al. Bovine viral diarrhea virus in alpaca: Abortion and persistent infection. J Vet Diagn Invest 2005;17:589–593.

14. Foster AP, Houlihan M, Higgins RJ, et al. BVD virus in a British alpaca. Vet Rec 2005;156:718–719.

15. Mattson DE, Baker RJ, Catania JE, et al. Persistent infection with bovine viral diarrhea virus in an alpaca. J Am Vet Med Assoc 2006;228:1762–1765.

16. Kelling CL, Steffen DJ, Topliff CL, et al. Comparative virulence of isolates of bovine viral diarrhea virus type II in experimentally inoculated six- to nine-month-old calves. Am J Vet Res 2002;63:1379–1384.

17. Kelling CL, Stine LC, Rump KK, et al. Investigation of bovine viral diarrhea virus infections in a range beef cattle herd. J Am Vet Med Assoc 1990;197:589–593.

18. Weiser MG, Fettman MJ, Van Houten D, et al. Characterization of erythrocytic indices and serum iron values in healthy llamas. Am J Vet Res 1992;53:1776–1779.

19. Carman S, van Dreumel T, Ridpath J, et al. Severe acute bovine viral diarrhea in Ontario, 1993–1995. J Vet Diagn Invest 1998;10:27–35.

20. Brodersen BW, Kelling CL. Effect of concurrent experimentally induced bovine respiratory syncytial virus and bovine viral diarrhea virus infection on respiratory tract and enteric diseases in calves. Am J Vet Res 1998;59:1423–1430.

21. Bezek DM, Mechor GD. Identification and eradication of bovine viral diarrhea virus in a persistently infected dairy herd. J Am Vet Med Assoc 1992;201:580–586.

22. Collins ME, Desport M, Brownlie J. Bovine viral diarrhea virus quasispecies during persistent infection. Virology 1999;259:85–98.

23. Munoz-Zanzi CA, Thurmond MC, Johnson WO, et al. Predicted ages of dairy calves when colostrum-derived bovine viral diarrhea virus antibodies would no longer offer protection against disease or interfere with vaccination. J Am Vet Med Assoc 2002; 221:678–685.

24. Ridpath JE, Neill JD, Endsley J, et al. Effect of passive immunity on the development of a protective immune response against bovine viral diarrhea virus in calves. Am J Vet Res 2003;64:65–69.

25. Kirkland P, McGowan M, Macintosh S. Factors influencing the development of persistent infection in cattle with pestivirus. In: The Second Symposium on Pestiviruses, Lyon, France, 1993;17–21.

26. Edmondson MA, Walz PH, Johnson JW, et al. Experimental exposure of pregnant alpacas to different genotypes of bovine viral diarrhea virus. 4th US BVDV Symposium, Phoenix, AZ, 2009.

27. Johnson JW, Edmondson MA, Walz PH, et al. Experimental exposure of naive alpacas to different genotypes of bovine viral diarrhea virus isolated from cattle and alpacas. 4th US BVDV Symposium, Phoenix, AZ, 2009.

28. Piccinini R, Luzzago C, Frigerio M, et al. Comparison of blood non-specific immune parameters in bovine virus diarrhoea virus (BVDV) persistently infected and in immune heifers. J Vet Med B Infect Dis Vet Public Health 2006;53:62–67.

29. Archambault D, Beliveau C, Couture Y, et al. Clinical response and immunomodulation following experimental challenge of calves with type 2 noncytopathogenic bovine viral diarrhea virus. Vet Res 2000;31:215–227.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotypic appearance of persistently BVDV infected alpacas. (a) Normally developed 4 month old BVDV PI Huacaya alpaca cria with "suri-like" fleece. (b) Markedly stunted 2 month old BVDV PI Huacaya alpaca cria. (c) Normally developed 10 month old BVDV PI Huacaya alpaca. (d) Mildly stunted 10 month old BVDV PI Huacaya alpaca. (e) Large-sized $3\frac{1}{2}$ year old BVDV PI Huacaya alpaca [matured alpaca (c)]. (f) Stunted $3\frac{1}{2}$ year old BVDV PI Huacaya alpaca with chronic dermatitis (white facial discoloration) [matured alpaca (d)].

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