



## **Diagnostic Testing at GeneSeek, Inc. for Detection of Bovine Viral Diarrhea Virus in alpaca**

### **The virus**

Bovine viral diarrhea virus (BVDV) is a single-stranded positive-sense RNA virus and is classified in the genus *Pestivirus* in the family *Flaviviridae*. In cattle, BVDV causes a variety of clinical symptoms, most notably reproductive failure through infertility, abortions, still births or births of weak calves, and/or calves with congenital defects. A hallmark of BVDV pathogenesis is the ability of the virus to establish persistent infection of the fetus when a naive pregnant dam is exposed to the virus during the first trimester of pregnancy. At this time, the bovine fetus is not developed far enough to recognize the virus as invader. Those calves are then born with a high titer of virus at birth and are unable to clear the infection over the course of their entire lives. Transiently, the virus may be neutralized by maternal antibodies passed on with colostrum and milk but viremia returns as maternal antibody concentration diminishes. PI animals continuously shed the virus from their mucosal surfaces and it is currently well accepted that direct contact with these persistently infected (PI) animals is the primary mode of BVDV transmission within and among herds. On the basis of a particular genome sequence, distinct genotypes of BVDV are recognized; BVDV type 1 with subtypes 1a and 1b is most common. More recently, type 2 and type 2 subtypes have been described.

### **The host**

Since the first description of BVDV in cattle in 1946, the virus has been documented to infect various other species (including goats, sheep, swine, deer, alpacas, and llamas). While BVDV infection and disease pathogenesis in cattle has been the subject of intense research efforts for many years, it has only been recently that disease associated with BVDV infections in New World camelids (alpacas and llamas) has been described in the literature. Early reports<sup>11</sup> mention serological evidence of BVDV exposure, i.e. the presence of BVDV antibodies, but clinical disease had not been recognized and seroprevalence was believed to be low<sup>9</sup>. Experimental inoculation of four pregnant llamas could not produce a persistently infected cria and clinical disease was not observed<sup>13</sup>.

Currently, however, accumulating evidence suggests that BVDV is a significant emerging disease in alpacas. It is now recognized that BVDV can cause a variety of clinical signs from vague signs of illness to death. Clinical manifestations include chronic ill thrift, anorexia, lethargy, diarrhea, stillbirths, abortions, births of underweight crias, congenital defects, and respiratory distress<sup>1,2,3,5,6,10</sup>. Exposure of a pregnant alpaca to BVDV during a critical time in the first trimester of gestation results in the birth of persistently infected crias<sup>3,10,2,12</sup>. Even though

the BVDV incidence in the North American alpaca herds appears to be low<sup>8</sup>, economic losses to producers due to BVDV may be substantial because of the significant value of the individual animal. While exposure to BVDV may be a rare event, the industry's habit of extensive traveling with susceptible females (with a cria at foot) for breeding and shows has been implemented in the spread of BVDV<sup>8,12</sup>.

### **Methods of testing**

There is a variety of routine diagnostic tests available for the detection of BVDV or detection of exposure to BVDV since the virus has been endemic in the US cattle population for several decades. In general, the diagnostic tests used for BVDV detection in cattle are well suitable for detection of BVDV in alpaca<sup>3</sup>.

**The Polymerase Chain Reaction (PCR)** detects the RNA of the virus. It does not distinguish between live or dead virus. It is exquisitely sensitive and does not take long to complete. RNA viruses, however, mutate at a high rate and it is possible that the PCR cannot detect the RNA anymore once critical PCR recognition regions of the virus have mutated.

**Virus Isolation (VI)** uses a culture of BVDV susceptible cells to make virus present in a sample grow. Cytopathic virus will destroy the cells as it grows, i.e. will have a cytopathic effect. The presence of non-cytopathic virus has to be demonstrated by using specific antibodies. Virus isolation works only with live virus so good sample quality is essential. Virus isolation takes several days to complete.

**Enzyme Linked Immunosorbent Assay (ELISA)** can demonstrate the presence of BVDV by using specific antibodies that bind to most all known BVDV types and strains. The commercial ELISA assay used routinely in cattle diagnostics, however, is not licensed for use in alpaca samples. Since the BVDV titer in alpacas is assumed to be lower than in cattle (Mattson 1994), the ELISA may not be sensitive enough to be a useful diagnostic test for alpacas.

**Immunohistochemistry (IHC)** is the gold standard diagnostic test for confirmation of PI status in cattle. This test looks for the presence of BVDV virus in tissues, most commonly in an ear notch. PI animals have the virus in their tissues while acutely infected animals generally harbor the virus only in the blood, i.e. are viremic, but not in any tissues. This appears to be the case for alpaca PI animals also.

**Serum neutralization assay (SN test)** looks for the presence of antibodies in serum. It is believed that alpaca that get infected with BVDV only have a brief period of viremia (followed by the production of neutralizing antibodies that eliminate the virus from the blood). The presence of antibodies is, therefore, indicative of exposure of an animal to BVDV even after the virus is no longer detectable.

All tests are proven to be sensitive and specific for detection of either BVDV RNA (PCR), the virus itself (IHC, antigen ELISA, IHC), or specific antibodies (SN). Depending on the objective of testing, therefore, several tests are available.

### **Validation of BVDV testing at GeneSeek**

GeneSeek is ISO17025 accredited through A2LA (<http://www.a2la.org>), based on internationally accepted criteria for quality and competence of testing. BVDV testing is in the scope of tests accredited and validation of testing is an integral part of our commitment to quality. GeneSeek's research and development for BVDV testing in alpacas by ELISA and PCR started in June 2006. A suitable method for viral RNA extraction has since been developed. Currently, GeneSeek

conducts testing using the PCR method on whole blood samples that have been spotted on blood cards. The objective of our validation was to confirm, with known positive samples of various BVDV concentrations, our ability to reliably detect the presence of BVDV RNA on blood cards from alpacas.

The BVDV serum titers of three confirmed persistently BVDV infected alpacas were determined by a TCID<sub>50</sub> assay: each serum was 10fold serially diluted and 4 replicate cell culture wells were inoculated with each dilution. From the highest dilution of sample that still infects 50% of the cell culture wells (2 of 4 cell culture wells) the serum titer is determined with a formula and expressed in tissue culture infectious dose per milliliter serum (TCID<sub>50</sub>/ml) as log<sub>10</sub>. The TCID<sub>50</sub>/ml indicates how much live virus is in a sample.

The whole blood from each animal was then serially diluted with BVDV-PCR negative alpaca blood and all samples (as is and all dilutions) spotted on blood cards. All blood cards were then processed using GeneSeek's proprietary standard operating procedures to extract viral RNA from blood cards and conduct the BVDV PCR.

## Results:

animal1	titers*	BVDV detected?	animal2	titers*	BVDV detected?	animal3	titers*	BVDV detected?
as is	7.1x10 <sup>4</sup>	YES	as is	6.3x10 <sup>5</sup>	YES	as is	3.5 x 10 <sup>4</sup>	YES
1 (10-fold)	7.1x10 <sup>3</sup>	YES	1 (10-fold)	6.3x10 <sup>4</sup>	YES	1 (10-fold)	3.5 x 10 <sup>3</sup>	YES
2 (5-fold)	2.1x10 <sup>3</sup>	YES	2 (5-fold)	1.3x10 <sup>4</sup>	YES	2 (5-fold)	8.5 x 10 <sup>2</sup>	YES
3 (5-fold)	7.1x10 <sup>2</sup>	YES	3 (5-fold)	6.3x10 <sup>3</sup>	YES	3 (5-fold)	3.5 x 10 <sup>2</sup>	YES
4 (5-fold)	2.1x10 <sup>2</sup>	YES	4 (5-fold)	1.3x10 <sup>3</sup>	YES	4 (5-fold)	8.5 x 10 <sup>1</sup>	YES
5 (5-fold)	7.1x10 <sup>1</sup>	YES	5 (5-fold)	6.3x10 <sup>2</sup>	YES	5 (5-fold)	3.5 x 10 <sup>1</sup>	no
6 (5-fold)	2.1x10 <sup>1</sup>	YES	6 (5-fold)	1.3x10 <sup>2</sup>	no	6 (5-fold)	8.5 x 10 <sup>0</sup>	no
7 (5-fold)	7.1x10 <sup>0</sup>	YES	7 (5-fold)	6.3x10 <sup>1</sup>	YES	7 (5-fold)	3.5 x 10 <sup>0</sup>	no
8 (5-fold)	2.1x10 <sup>0</sup>	no	8 (5-fold)	1.3x10 <sup>1</sup>	no	8 (5-fold)	8.5 x 10 <sup>-1</sup>	no
9 (5-fold)	7.1x10 <sup>-1</sup>	no	9 (5-fold)	6.3x10 <sup>0</sup>	no	9 (5-fold)	3.5 x 10 <sup>-1</sup>	no

\*titers are expressed in TCID<sub>50</sub>/ml of serum

The data from this validation show that the methods employed readily detected field samples from BVDV-PI alpacas. The original titers of these animals ranged from 3.5x10<sup>4</sup> to 6.3x10<sup>5</sup> TCID<sub>50</sub>/ml. This is lower than the typical titer in BVDV-PI calves but much above the detection threshold of the PCR. The result 'BVDV detected, YES' indicates that the PCR has detected the presence of BVDV RNA in the sample. Virus loads of approximately 10<sup>2</sup> TCID<sub>50</sub>/ml were detected reliably; some lower titers were still detected. In samples from animal 2, the theoretical titer of 1.3x10<sup>2</sup> was not detected, however the titer resulting from another 5-fold dilution was detected. This irregularity is likely due to a mixing error while making the dilutions with whole blood.

No determination of status (persistently infected versus acutely infected) can be made at this time because the virus is present in the blood in both acutely and persistently infected animals. Careful follow-up testing is necessary over a period of several weeks to determine whether the presence of BVDV in the blood is persistent or transient. A BVDV positive re-test by PCR or VI after 4 weeks is generally assumed to be indicative of a persistent infection<sup>8</sup>.

## References:

1. Belknap EB, Collins JK, Larsen RS, and Conrad KP (2000) Bovine viral diarrhea virus in New World camelids. *J Vet Diagn Invest* 12:568-570
2. Byers SR, Snekvik KR, Righter DJ, Evermann EF, Bradway DS, Parish SM, Barrington GM (2009) disseminated Bovine viral diarrhea virus in a persistently infected alpaca (*vicugna pacos*) cria. *J Vet Diagn Invest* 21:145-148
3. Carman S, Carr N, DeLay J, Baxi M, Deregt D, and Hazlett M (2005) Bovine viral diarrhea virus in alpaca: abortion and persistent infection. *J Vet Diagn Invest* 17:589-593
4. Evermann JF (2006) Pestiviral infection of llamas and alpacas. *Small Rumin Res* 61(2):201-206
5. Foster AP, Houlihan MG, Holmes JP, Watt EJ, Higgins RJ, Errington J, Ibata G, and Wakely PR (2007) Bovine viral diarrhea virus infection of alpacas (*Vicugna pacos*) in the UK. *Vet Rec* 161:94-99)
6. Goyal SM, Bouljihad M, Haugerud S, and Ridpath JF (2002) Isolation of bovine viral diarrhea virus from an alpaca. *J Vet Diagn Invest* 14:523-525
7. Kapil S, Yeary T, Evermann JF (2009) Viral Diseases of New World Camelids. *Vet Clin Food Anim* 25:323-337
8. Kim SG, Anderson RR, Yu JZ, Zylich NC, Kinde H, Carman S, Bedenice D, and Dubovi EJ (2009) Genotyping and phylogenetic analysis of bovine viral diarrhea virus isolates from BVDV infected alpacas in North America. *Vet. Microbiol.* 136 (3-4):2009-2016
9. Mattson DE (1994) Viral diseases update on Llama medicine. *Vet Clin North Am Large Anim Pract* 10:345-341
10. Mattson DE, Baker RJ, Catania JE, Imbur SR, Wellejus KM, and Bell RB (2006) Persistent infection with bovine viral diarrhea virus in an alpaca. *J Am Vet Med Assoc* 228(11):1762-1765
11. Thedford TR, and Johnson LW (1989) Infectious diseases of New-World camelids (NWC) *Vet Clin North Am Food Anim Pract* 5(1):145-157
12. Topliff CL, Smith DR, Clowser SL, Steffen DJ, Henningson JN, Brodersen BW, Bedenice D, Callan RJ, Reggiardo C, Kurth KL, and Kelling CL (2009) Prevalence of bovine viral diarrhea virus infections in alpacas in the United States. *J Am Vet Med Assoc* 234:519-529
13. Wenz PA, Belknap EB, Brock KV, Collins JK, and Pugh DG (2003) Evaluation of bovine viral diarrhea virus in New World camelids. *J Am Vet Med Assoc* 223(2):223-228