BOVINE VIRAL DIARRHEA VIRUS: EVALUATION OF PERSISTENT INFECTIONS, ACUTE TRANSMISSION, AND VACCINATION PROTECTION IN ALPACAS

By

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A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTERS OF SCIENCE IN VETERINARY SCIENCE

WASHINGTON STATE UNIVERSITY
College of Veterinary Medicine
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MAY 2009

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ACKNOWLEDGEMENT

I would like to express my sincere appreciation to my committee chair Dr. George Barrington for his patience, assistance, encouragement, and great advice. I also want to thank my committee members for all their assistance and support in completing this endeavor: Dr. Evermann for his expertise, enthusiasm, and always appearing to be happy to see me knocking on his door; Dr. Tibary for his advice on the projects, assistance with the camels, and obtaining research animals for my projects; and Dr. Parish for his support and encouragement and if not for his persistence and persuasion there would not have been a residency position for me.

Finally, I can not begin to express my gratitude to my husband Thomas, who has never stopped encouraging and supporting me. I can not imagine making this journey without him.

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Abstract

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May 2009

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The purpose of this project was to characterize bovine viral diarrhea virus (BVDV) type 1b infection in alpacas to determine the effects of persistent infection (PI), acute transient disease, and vaccine efficacy. The first specific aim was designed to follow three PI alpacas from identification of the BVDV infection through the end of life. Antemortem and postmortem testing identified viral shedding patterns and organs affected by virus. Results indicated variability in antigen location between alpacas; however, viremia and viral shedding was consistent in all three PI alpacas until death. Specific aim two characterized BVDV transmission and acute infection in previously unexposed alpacas. The study involved two groups of alpacas, one experimentally inoculated with BVDV, and the second housed with PI alpacas to facilitate direct, natural transmission. Clinical signs in the previously unexposed alpacas were mild, although alterations in blood cell parameters indicated immunosuppression similar to that seen in bovidae infected with BVDV. The third specific aim evaluated the efficacy of a commercial, modified-live, BVDV vaccine in alpacas. Five previously unexposed alpacas were vaccinated and their serologic antibody response was documented. The five vaccinated and two unvaccinated

controls were challenged by nasal and ocular inoculation with BVDV Type 1b obtained from a PI alpaca. Vaccinated alpacas experienced no adverse side effects and failed to become viremic whereas the unvaccinated control alpacas developed viremia and experienced mild signs of acute BVDV infections. The vaccine appeared efficacious for use in nonpregnant alpacas.

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CHAPTER 1

INTRODUCTION

This thesis consists of an introduction, a literature review, a summary on three persistently infected alpacas, four manuscripts submitted for publication, a conclusion, and appendices. This graduate student was the principle contributor and author of this thesis and manuscripts. The manuscripts have been reviewed by the committee members. Their comments and advice have been considered and changed made where deemed appropriate. All final changes made were the decision of this graduate student.

Bovine viral diarrhea virus (BVDV) primarily affects cattle however it can infect other even-toed ungulates such as alpacas. In recent years, there have been an increasing number of reports of clinical disease and persistent infections similar to that observed in cattle. The cause of the sudden increase in clinical BVDV in alpacas has not been identified but is thought to be related to the mutagenic aspects of the virus. Since clinical BVDV has only recently emerged in alpacas, there is a paucity of information about all aspects of the disease in this species. Some information can be extrapolated from research in the bovine species but aspects such as diagnostic methods, transmission, and protection need to be verified in alpacas.

The purpose of this study is to characterize BVDV in alpacas to determine persistent infection consequences, transmission modes, disease effects, and vaccine protection. I hypothesize that the clinical disease and transmission of BVDV in alpacas is comparable to that of cattle and a commercial bovine vaccine protects against experimental infection.

Chapter 2 is a literature review of BVDV in alpacas including pertinent research information from the bovine species.

Chapters 3 and 4 summarize the information learned from three persistently infected (PI) alpacas. The alpacas were verified to be PI on a monthly basis until death. Various bovine diagnostic tests including polymerase chain reaction (PCR), virus isolation (VI), antigen capture enzyme-linked immunosorbent assay (AgELISA), and serum neutralization (SN) were verified for use in alpacas. All three alpacas eventually succumbed to secondary bacterial infections. Postmortem testing found variations in tissue infectivity. The severity of clinical illness discovered on postmortem testing corresponded to the degree of BVDV infection detected in each alpaca, with the least severe signs and longest life span in the alpaca with minimal antigen presence in tissues. All three alpacas had viral antigen present in saliva and nasal secretions and urine, two had antigen present in gastrointestinal tissues, the female had viral antigen detected in uterine tissues and feces, and one of the two males had antigen present in testicular tissues. The results indicated all body fluids were potential shedding routes for transmission to other alpacas. The study on the first PI alpaca to succumb was published in the Journal of Veterinary Diagnostic Investigations and is found in Chapter 4.

Chapters 5 and 6 summarize the results from a preliminary project performed to evaluate the effects on alpacas experimentally inoculated with a BVDV strain from a PI alpaca, followed by a natural transmission study using two of the PI alpacas described above. The preliminary study found minimal signs of disease following intravenous and intrarectal inoculation. All five inoculated alpacas became viremic

and virus was detected in salivary gland and saliva of two of the alpacas. The natural transmission study was then performed with six alpacas to evaluate methods of infection, shedding, and duration of clinical signs following exposure to PI alpacas. All six alpacas became viremic however were not found to shed virus in nasal or oral secretions. Clinical signs were minimal. Transmission occurred primarily by the respiratory route since the direct contact and fence-line contact animals became infected. The experimental inoculation study will be submitted to Veterinary Pathology and the natural transmission study has been submitted to the Canadian Veterinary Journal.

Chapter 7 describes the results from the BVDV vaccination trial. Five alpacas were vaccinated with a commercial modified-live BVDV vaccine commonly used in cattle. Two other alpacas were not vaccinated and all seven were inoculated by the intranasal route with BVDV cultured from one of the PI alpacas discussed above. The PI alpaca succumbed prior to the start of this phase of the project so direct challenge could not be performed. The vaccinated alpacas did not become viremic or develop clinical signs of BVDV infection. The two unvaccinated alpacas developed viremia and signs of upper respiratory infections before serologic titers were detected and they cleared the viremia and respiratory infections. This study has been submitted to Veterinary Microbiology.

CHAPTER 2

LITERATURE REVIEW

Bovine viral diarrhea virus (BVDV) is one of several pestiviruses in the Flaviviridae family. It is related to Border Disease and Classical Swine Fever. Bovine are the primary species infected by BVDV, however research has identified it in most even-toed ruminants with varying degrees of disease produced.[1,2] Research on BVDV has been performed since it was first identified in cattle in 1946 and since then much has been identified about the virus and disease it produces in cattle.[3]

The two classification methods used to identify BVDV are genotype and biotype. Bovine viral diarrhea virus can be divided into two genotypes: BVDV type 1 and BVDV type 2. Type 1 BVDV is found worldwide whereas type 2 is found predominantly in North America.[4] Within each genotype are numerous subgenotypes indicating the dynamic genome and mutagenic feature of the virus. There are approximately 11 subtypes of BVDV type 1 and 2 subtypes of BVDV type 2.[5,6] The significance of the multiple subtypes is not well understood since virulence and disease is not only dependent on the subtype but also infectious dose, individual animal immune response, and environmental conditions. The numerous subtypes likely contribute to the failure of various vaccination control strategies and the development of over 100 commercial vaccines.[7]

Biotype refers to the cytopathic (cp) or noncytopathic (ncp) feature of the virus in cell cultures. Both type 1 and type 2 BVDV can have cp and ncp forms and both can be found in natural infections. Only the ncp forms are thought to be capable of

causing persistent infections in the fetus however cp forms have been identified in fetal tissues and serum.[4,8-10] A BVDV persistently infected (PI) bovine co-infected with a closely related cp subtype can develop a rare and fatal form of BVDV known as mucosal disease.[11]

Bovine viral diarrhea virus has the ability to cause multiple forms of disease: persistent infections, transient infections, and mucosal disease. Persistent infections can develop if the fetus is exposed to the virus prior to maturation of the fetal immune system at around 125 days gestation.[12] In these cases, the immune system does not recognize the virus as a foreign antigen and does not mount an immune response. These animals will have detectable virus by tests such as PCR, VI, AgELISA, and immunohistochemistry (IHC), but will be antibody negative to that strain of virus.

Transient infections occur in previously unexposed animals and are typically acute and subclinical in 70-90% of infected cattle.[13] However, severity does vary and can include respiratory disease, infertility, abortions, congenital defects, hemorrhagic disorders, and even death in severe cases.[12,14] Mucosal disease occurs sporadically and is typically fatal due to the severe ulcerations of all mucosal surfaces and secondary infections.[12] Bovine viral diarrhea virus is also known to induce immune suppression leading to many secondary illnesses such as the respiratory disease complex commonly observed in feedlot cattle.[12,15]

Bovine viral diarrhea virus transmission occurs by separate but interrelated vertical and horizontal modes. Horizontal transmission of a pregnant bovine can lead to vertical transmission to the fetus causing a persistent infection. The PI animal is the primary source for horizontal transmission of BVDV in a herd since large amounts of

virus are shed in all body fluids, but transiently infected (TI) animals can also be a major source of infection.[16,17] Horizontal transmission occurs within one hour of direct contact with a PI animal and fence line transmission from both PI and TI animals has been reported.[18,19] Inhalation of virus-laden body fluids is thought to be the primary route of infection for acute infections.[20]

A variety of diagnostic tests are available and some are more suited for distinguishing the PI animal from the TI animal. Persistent infections can be detected using a combination of VI of the buffy coat layer or serum, IHC staining for viral antigen in skin, AgELISA of skin or blood samples, and PCR of whole blood or serum samples.[21,22] Tests such as VI or PCR cannot differentiate persistent from transient infections so retesting several weeks later in combination with serum neutralization is used.

Various control and eradication strategies have evolved over time and currently are based on testing and culling PI animals, vaccinations, and biosecurity. Detection of the PI animal is important as this animal is the primary source of infection within the herd. Even so, producers utilizing these control points continue to observe problems due to transient infections and vaccination or biosecurity failures.

Prior to 2005, very little research had been published evaluating BVDV in camelids. Only one research project evaluated BVDV inoculation in pregnant llamas.[23] Other reports indicated camelids formed antibodies to the virus but clinical signs were either absent or mild so the prevailing thought was the virus did not readily affect camelids.[24-27] In 2005, the first reports of suspected clinical BVDV cases and PI alpacas emerged.[28,29] At that time, most bovine diagnostic tests had not been validated

for use in alpacas so the initial cases of suspected PI alpaca crias were difficult to confirm. Diagnostic laboratories appeared to settle on PCR and serology as the primary methods to test alpacas.

Since 2005, other reports have been published indicating an increasing incidence of BVDV cases in alpacas.[30-33] Current research indicates the majority of BVDV cases in alpacas have been classified as type 1b but some type 2 cases have been detected.[28,29,31-35] Alpacas also have not exhibited the severe mucosal disease seen in cattle. Due to the recent emergence of BVDV in alpacas, most reports have only focused on detection, genetics, and prevalence so basic questions regarding transmission, duration of infection, shedding routes, clinical effects, and control mechanisms have not been well defined.

The purpose of this study was to determine the effects of BVDV persistent infections, transient infections, transmission, and vaccination protection in alpacas.

CHAPTER 3

EVALUATION OF PERSISTENTLY INFECTED ALPACA CRIAS

Abstract

Bovine viral diarrhea virus (BVDV) is an emerging infectious pathogen of concern to the alpaca industry. Three alpaca crias from a single farm were diagnosed as persistently infected with BVDV based on repeated positive antemortem PCR and VI assays, and negative antibody titers to BVDV. Various postmortem diagnostic tests were performed with virus identified in multiple tissues demonstrating disseminated BVDV type 1b infections in all three alpacas. Virus was detected in various body fluids indicated potential routes of shedding and transmission to other alpacas.

Introduction

Three alpacas crias: a 6-month-old female (WSU-D) and two approximately 5-month-old, intact males (WSU-S and WSU-R), presented to the Washington State University Veterinary Teaching Hospital (Pullman, WA) with a history of decreased weight gain, ill thrift, and anorexia. The alpacas were from a herd of 45 animals and diagnosed as PI alpacas infected with BVDV type 1, based on repeated positive tests by PCR and VI on EDTA blood samples obtained at monthly intervals staring at 2-3 months of age, and negative SN antibody titers on serum samples. Upon presentation, all three crias were bright, alert, and responsive with normal vital parameters (temperatures, pulse, and respiration), however all three were deemed to be significantly underweight compared to age-matched uninfected crias. Because of the

grave prognosis due to their PI status and general poor health, as well as financial considerations, the owners declined further antemortem diagnostics tests or treatment and elected to donate the alpaca crias to WSU for monitoring.

WSU-R

WSU-R was five months old and weighed 13.6 kg at presentation. Besides a small stature and poor body condition score (1.5/5), all other findings on physical examination were within normal limits. Complete blood cell count revealed a mild left shift (band neutrophils 309/µl; reference [ref.] range <200/µl), high normal fibrinogen (400 mg/dl; ref. range 100–400 gm/dl), anemia (PCV 21%, ref. range 27–45%), decreased hemoglobin (9.4 g/dl; ref. range 11.9–19.4 g/dl), and marked anisocytosis. Serum biochemical profile was within normal limits.

Therapy with tulathromycin (5.5 mg/kg intramuscular, Draxxin®, Pfizer Animal Health, New York, NY) and a nutritional supplement had been initiated prior to admittance to WSU due to a suspected upper respiratory tract infection. Over the next eight days at WSU, the cria was observed to be ambulating, eating, drinking, urinating, and defecating normally. Florfenicol therapy (22 mg/kg, subcutaneous, q 48 h; Nuflor®, Intervet/Schering-Plough, Union, NJ) was initiated on day 4 because of a suspected respiratory infection. On the morning of day 9, the cria was found dead in its stall and transferred to the Washington Animal Disease Diagnostic Laboratory (WADDL, Pullman, WA) for complete postmortem examination.

WSU-D

WSU-D was six months old and weighed 19.8 kg at presentation. Besides a small stature and poor body condition score (1.5/5), all other findings on physical examination were within normal limits. Complete blood cell count revealed a neutrophilia (segmented neutrophils 1501/µl; ref. range 4700-14900/µl), anemia (PCV 24%, ref. range 27–45%), decreased hemoglobin (10.4 g/dl; ref. range 11.9–19.4 g/dl), and moderate anisocytosis. Serum biochemical profile revealed an elevated SDH (32 U/L; ref. range 1-5 U/L), elevated AP (190 U/L; ref. range 27-132 U/L), mildly elevated CK (106 U/L; ref. range 8-77 U/L), mildly decreased total protein (5.0 g/dl; ref. range 5.1-7.8 g/dl) and mildly decreased albumin (3.0 g/dl; ref. range 3.1-5.2 g/dl). All other values were within normal limits.

Therapy with tulathromycin (5.5 mg/kg IM) had been initiated prior to admittance to WSU due to a suspected upper respiratory tract infection. Over the next three months of monitoring, the cria was observed to be ambulating, eating, drinking, urinating, and defecating normally. Florfenicol therapy (22 mg/kg, SC, q 48 h) was initiated on day 4 because of a suspected respiratory infection and chronic pyrexia. A recurrent fever occurred every few weeks for 4-8 days at a time and ranged from 102.8-103.8°F. Antipyretics were not administered as the cria remained bright and appetent. The febrile episodes would typically be accompanied by nasal and or ocular discharge consistent with an upper respiratory tract infection. Due to the persistent respiratory tract signs, the cria remained on florfenicol for approximately three weeks before therapy was changed to ceftiofur crystalline free acid (6.6 mg CE/kg SC, q 7 d; Excede, Pfizer Inc., New York, NY). Monthly sampling was performed to monitor

BVDV PI status and shedding (Table 1) and CBC values (Table 2). Three months after arrival, the cria was found to have a sinus abscess that eroded through the frontal bone. Due the cria's continued deterioration and immune suppression, it was euthanized and transferred to WADDL for complete postmortem examination.

WSU-S

WSU-S was 4-1/2 months old and weighed 15.5 kg at presentation. Besides a small stature, and poor body condition score (2.0/5), all other findings on physical examination were within normal limits. Blood work was performed the day after arrival at WSU. Complete blood cell count revealed only mild changes including a lymphocytosis (6302/µl; ref. range 700-4800/µl), high normal fibrinogen (400 mg/dl; ref. range 100–400 gm/dl), and marked anisocytosis with slight to moderate polychromasia. Serum biochemical profile revealed mild increases in GGT (51 U/L; ref. range 9-29 U/L), ALP (468 U/L; ref. range 27-132 U/L), and CK (246 U/L; ref. range 8-77 U/L).

Over the next 11 months of monitoring, the cria was observed on a daily basis with only mild intermittent health problems. Florfenicol therapy (22 mg/kg, SC, q 48 h) was initiated on day 4 because of suspected respiratory infections in the two other PI crias housed with this animal and potential BVDV induced immune suppression in conjunction with shipping and environmental stresses. The cria did not develop signs of respiratory disease similar to the other two PI crias until just prior to death. The alpaca was relatively healthy with minor illnesses including a corneal ulcer due to a foreign body and a chronic hyperplasia along the oral mucocutaneous junction.

Monthly sampling was performed to monitor BVDV PI status and shedding (Table 1) and CBC values (Table 3). At 15 months old, the cria developed a respiratory tract infection with anorexia and suddenly died while undergoing medical treatment to stabilize its condition. The cria was transferred to the WADDL for complete postmortem examination.

Diagnostic Testing and Results

WSU-R

Gross postmortem examination revealed acute, locally extensive bronchopneumonia of the cranial lung lobes; caudal lung lobes were mottled red to pink and moderately firm. Several well delineated (0.5–1.5 cm in diameter) ulcers were observed on the mucosal surface of gastric compartments 1 (C1), 2 (C2), and 3 (C3). Numerous petechiae were noted on the surface of the spleen. No other gross lesions were identified. Tissues were collected in 10% neutral buffered formalin, routinely processed, and embedded in paraffin blocks. From the paraffin blocks, 4-µm sections were cut and stained with hematoxylin and eosin. Lung, liver, and a tracheal swab submitted for aerobic culture revealed mixed bacterial growth; similar samples were negative for *Mycoplasma* culture.

Histological examination of tissue from the cranial lung lobes revealed moderate, multifocal, suppurative bronchopneumonia. Sections from the remaining lung lobes were characterized by patchy infiltrates of minimal to low numbers of lymphocytes, plasma cells, and rare neutrophils within the interlobular and alveolar septa. Gastric compartments 1 and 2 had multifocal gastritis, characterized by a mild

to moderate infiltration of the lamina propria by variable admixtures of lymphocytes, plasma cells, eosinophils, neutrophils, and macrophages. No fungi were identified. In several sections of both large and small intestine, the lamina propria was mildly to moderately expanded by neutrophils, lymphocytes, and plasma cells. The liver exhibited moderate, multifocal, random hepatocellular necrosis consistent with bacteremia. Submandibular, inguinal, and mesenteric lymph nodes had mildly to markedly reduced numbers of lymphocytes within the cortex and medulla; mesenteric lymph nodes were most severely affected. The cortex and medulla of the thymic lobules and the white pulp of the spleen had moderately to severely reduced lymphocyte numbers; the thymic corticomedullary junction was indistinct.

Immunohistochemical (IHC) detection of BVDV antigen was performed on sections of formalin-fixed, paraffin-embedded tissues using monoclonal antibody (mAb) 15c5 (IDEXX MoAB 15c-5 (anti-BVDV EO), IDEXX Laboratories, Westbrook, ME, USA) at a 1:1000 dilution in a streptavidin-biotin-immunoperoxidase technique with diaminobenzidine as a chromogen (LSAB™2 Kit, Dako North America Inc., Carpinteria, CA, USA).[36] Large amounts of BVDV antigen were detected in the following tissues: parotid salivary gland, testis, prostate, esophagus, C1, C2, C3, right kidney, bone marrow, liver, lung, spleen, thymus, and the mesenteric and submandibular lymph nodes. In sections of parotid salivary gland, there was strong intracytoplasmic immunoreactivity within acinar epithelial cells and occasional ductular epithelial cells. Similarly, a section of testis revealed frequent cells within the seminiferous tubules that had strong intracytoplasmic immunoreactivity for BVDV antigen. Scattered groups of epithelial cells within the convoluted tubules of the

kidney had mild to moderate intracytoplasmic immunoreactivity for BVDV, while rare macrophages within the interstitium had intracytoplasmic immunoreactivity for BVDV. Within the esophagus and C1, there was BVDV immunoreactivity within the cytoplasm of basaloid epithelial cells. Compartment 2 had immunoreactivity of both the basaloid epithelial cells within the areas of stratified squamous epithelium and within the glandular epithelium of the saccules. In C3, there were frequent areas of immunoreactivity within the cytoplasm of glandular epithelial cells and within some of the gastric ulcers. Within the lung, thymus, spleen, and lymph nodes, there were rare to frequent cells (presumptive macrophages) that had intracytoplasmic immunoreactivity for BVDV antigen, while scattered Kupffer cells within the liver had similar immunoreactivity. Frequent myeloid precursor cells and presumptive macrophages within the bone marrow had similar intracytoplasmic immunoreactivity for BVDV antigen.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to detect and subtype BVDV in tissues was performed using a duplex TaqMan real-time PCR procedure as previously described.[37] Following extraction of total RNA with TRIzol (Invitrogen Corp., Carlsbad, CA, USA), BVDV-1 was detected in kidney, liver, spleen, lung, and thymus. Bovine viral diarrhea virus was isolated from the same tissues in BVDV-free bovine turbinate cells.[22] Bovine viral diarrhea virus RNA was extracted from the supernatants of virus-infected cells using a commercial RNA extraction kit (QIAamp® Viral RNA Mini Kit, Qiagen Inc., Valencia, CA, USA) and detected via TaqMan real-time PCR procedure as previously described.[37] To obtain a longer amplicon for sequencing, a separate RT-PCR reaction was run, using

recombinant *Thermus thermophilus* (rTth) DNA polymerase (Applied Biosystems, Foster City, CA, USA) to amplify a highly conserved 290-bp portion of the 5'-untranslated region (5' UTR) of the BVDV genome as previously described.[38] Polymerase chain reaction amplicons were visualized on a 1.5% agarose gel containing ethidium bromide, excised using a sterile scalpel blade under ultraviolet illumination, and purified using a nucleic acid purification kit (Bio-Rad Freeze 'N Squeeze™ Kit, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's directions. Amplicon DNA was sequenced directly on both strands by a local vendor (Amplicon Express Inc., Pullman, WA, USA) using a commercial sequencing kit (Amersham DYEnamic ET Terminator Cycle Sequencing Kit, Amersham Biosciences, Piscataway, NJ, USA) with analysis on a DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing reactions were done in duplicate, and sequences were confirmed by sequencing of both strands. Forward and reverse sequences were aligned using the ClustalW algorithm.[39] Each sequence was compared to the GenBank nucleotide sequence database for similarity using BLASTn (nucleotide Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence most closely matched that of BVDV-1b (98% sequence identity to GenBank accession # AY159530 and other type 1b accessions) when compared with sequences in GenBank.

WSU-D

Antemortem samples collected from WSU-D and tested as described above verified constant BVDV PI status and viral shedding in oral, nasal, fecal, and urine

samples with PCR, VI, and AgELISA (Table 1). Skin biopsy samples collected from ear notching were positive by the commonly used bovine AgELISA test. The alpaca was consistently negative on serology. Postmortem samples were collected and processed as describe above. Whole blood samples were collected at 7 and 9 months of age for flow cytometry (see Chapter 6 for processing details) and processed with llama specific monoclonal antibodies to determine percentages of leukocytes (Table 4). Cell population percentages were variable between both samples and were generally within the normal expected values for juvenile alpacas (Table 5).

Gross postmortem examination revealed thymic atrophy with scant amounts of ill defined thymic tissue in the mediastinal and distal cervical region. At approximately midline overlying the frontal sinuses there was a 1 cm circular ulceration of the skin with caseous to mucopurulent discharge with a draining inflammatory tract extended into the subjacent bone. An abundant amount of ale yellow purulent material was present within the frontal sinuses and the nasal cavity. Diffusely within the wall of C2, there were accumulations of edema fluid that expanded the wall up to 1 cm in thickness. Compartment 3 had a focal, 1.5 x 0.5 cm dark red ulcer overlaid by a 0.5 cm fibrinous pseudomembrane. The mucosa of the urinary bladder was stippled by numerous petechia. No other gross lesions were identified. Bacterial cultures of swabs taken from the trachea and nasal exudates revealed *Bordetella bronchiseptica* and *Arcanobacterium pyogenes*. Lung and liver swabs revealed primary growth of Bordetella bronchiseptica. Samples were negative for Mycoplasma culture.

Histological examination of tissue from sections of tonsil, thymus, spleen and submandibular, cervical, mesenteric and inguinal lymph nodes had generalized, moderate to severe lymphocyte depletion. Occasional lymphoid cortical follicles and splenic white pulp were admixed with pyknotic nuclear debris (lympholysis). Lymph node medullary cords were further severely depleted of macrophages, plasma cells and lymphocytes. Subcapsular and medullary sinuses of the submandibular lymph node were multifocally expanded by numerous degenerate neutrophils with fewer macrophages interspersed with flocculent eosinophilic proteinaceous material. The liver exhibited rare, multifocal hepatocellular necrosis. Gastric compartment 3 had focal ulcerative gastritis, characterized by lamina propria that was replaced by a coagulum of serocellular debris admixed with numerous bacterial colonies and degenerate neutrophils. The underlying submucosa was expanded by a mixture of neutrophils, lymphocytes, macrophages, and eosinophils interspersed with fibrin and edema residue. Replicate sections stained with Brown & Hopps tissue gram stain revealed large numbers of long slender gram negative rods along the advancing edge of the ulcerated mucosa. The uterus had suppurative, mild, diffuse, acute endometritis with scattered neutrophils, lymphocytes, and decreased plasma cells. Several large rafts of neutrophils are free within the uterine lumen. Sections of the lung lobes showed moderate, multifocal, peracute pulmonary congestion and atelectasis. The brain had a single vessel within the cerebral cortex bordered by mildly increased numbers of lymphocytes. The pulmonary changes detected grossly were attributed to multifocal congestion and atelectasis with agonal aspiration of mucocellular debris from the upper respiratory tract. The hepatic changes were speculated to be secondary

to septicemia or endotoxemia from the ulcerative gastritis. Suppurative endometritis was regarded as an incidental finding likely secondary to immunosuppression.

Immunohistochemical detection of BVDV antigen was performed as described above. Numerous skin and pancreatic acinar epithelial cells had strongly positive cytoplasmic immunoreactivity. Parotid salivary gland acinar and ductal epithelial cells comprised approximately 5% of the section, and were stippled with strongly positive cytoplasmic immunoreactivity. Polymerase chain reaction testing was positive for BVDV on lung, tracheal and nasal exudate swabs, whole blood and serum, feces, kidney, spleen, and thymus. Virus isolation was positive for BVDV on lung, kidney, spleen, thymus, and liver. Antigen ELISA was positive for BVDV on serum. The alpaca was serology negative for BVDV. The fecal sample also indicated a large population of giardia.

WSU-S

Antemortem samples collected from WSU-S and tested as described above verified constant BVDV PI status and viral shedding in oral, nasal, and urine samples with PCR, VI, and AgELISA (Table 1). Skin biopsy samples collected from ear notching were positive by the commonly used bovine AgELISA test. The alpaca was consistently negative on serology. Whole blood samples were obtained at 5, 7, and 11 months of age for flow cytometric analysis. Values were variable between the three samples and were generally within the normal reference values (Table 5). Postmortem samples were collected and processed as describe above.

Gross postmortem examination revealed severe consolidated and hyperemia of the right cranioventral lung lobes which affected approximately 20% of the tissue. On cut section, medium sized airways exuded green feed material. The left caudoventral lobes had irregular zones of consolidation affecting approximately 5% of the left lung. Sections of affected lung lobes did not float in 10% formalin. Tracheobronchial lymph nodes were moderately to severely enlarged. Lung and tissue pool samples submitted for aerobic culture revealed a mixed bacterial growth.

Histologic examination of lung tissue had broad regions with dense aggregates of degenerate neutrophils, cellular debris, colonies of mixed bacteria, and fragments of plant material filling bronchi and bronchioles. Adjacent alveoli were frequently filled with neutrophils and fewer macrophages admixed with large numbers of mixed bacteria. The trachea had a mild infiltration of lymphocytes and plasma cells within the subepithelial stroma. Many portal areas of the liver were surrounded by disorganized, proliferative bile ducts and biliary epithelial cells that did not form recognizable ductules. The adrenal gland contained scattered cortical foci expanded by mild hemorrhage. Several lymph node sections contained moderate numbers of neutrophils within the medullary sinuses and moderate numbers of macrophages that contain phagocytosed erythrocytes. The spleen had mild depletion of lymphocytes within the white pulp and numerous macrophages in the red pulp contained phagocytosed erythrocytes. The small intestinal villi in several segments were moderately to severely blunted and fused. The lamina propria was infiltrated with minimal numbers of lymphocytes and plasma cells.

Immunohistochemical detection of BVDV antigen was performed as described above. Only skin epithelial cells showed signs of positive cytoplasmic immunoreactivity.

Discussion

The results of the diagnostic testing indicated disseminated BVDV infections in all three alpacas. Interestingly, there was significant variation between the three animals as to which tissues were affected. Alpaca WSU-S had the least amount of detectable virus in the tissue samples and this alpaca also had the fewest clinical signs and survived the longest. The variation in presence of virus is may be due in part to different gestational infection time frames and is similar to that reported in other PI alpacas.[28,30-32,34] The exact time frame for development of persistent infections in alpacas has not yet been identified but is suspected to be in the first trimester as is reported in cattle. The dams of these three alpacas were all exposed to an acute or persistently infected alpaca at the breeding farm prior to being shipped home at 4-5 months pregnant.

Diagnosis of the PI status was verified consistently with positive PCR, VI, AgELISA, and negative serology. Viral antigen was detected in nasal and oral swabs, urine or urinary bladder, testicles, and gastrointestinal tract tissue or feces, which indicates potential routes of transmission similar to that observed in cattle. Flow cytometry results from WSU-S and WSU-D were generally within the normal expected range for healthy juvenile alpacas. Similar findings have been reported in PI cattle with lymphocytes typically within normal ranges.[40] All the PI alpacas in this

report were stunted and unable to gain weight, had poor fiber quality, and were predisposed to secondary infections to which they all eventually succumbed.

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Figure 1. Phylogenetic analysis and relatedness of the BVDV persistently infected alpaca crias to other known BVDV strains. Persistently infected alpacas are identified as WSU alpaca S, WSU alpaca R, and WSU alpaca D.

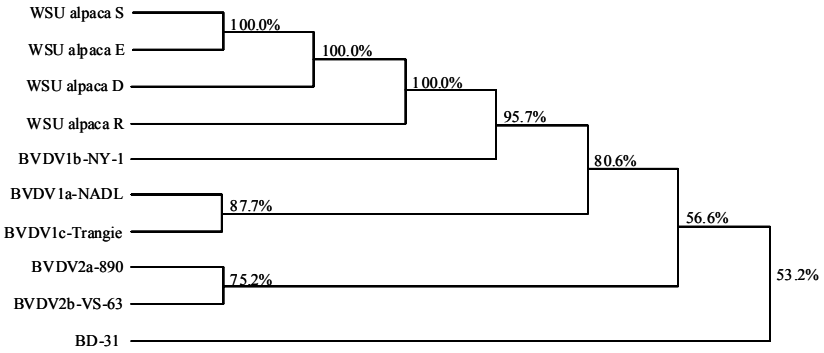

Table 1. Monthly testing to verify BVDV persistent infection status on WSU-R, WSU-D, and WSU-S. Time 0 for month tested indicates initial testing on arrival at WSU. Tests with spaces not containing '+' or '-' indicates test not performed. Oral and nasal samples were obtained by swabbing buccal and nasal mucosal surfaces.

Month Tested	WSU Alpaca	Sample	PCR	VI	AgELISA	Serology
0	R, D, S	blood	+	+	+	-
1	D, S	blood	+	+	+	-
		oral & nasal	+	+		
2	D, S	blood	+			-
3	D, S	blood	+			-
		ear notch			+	
4	D	feces, urine	+	+		
	D, S	blood	+			-
5	S	blood	+		+	-
6	S	blood	+			-
		oral & nasal	+	+		
7	S	blood	+			-
8	S	blood	+			-
9	S	blood	+			-
		oral & nasal	-	-		
10	S	blood	+	+		-
		urine, oral, nasal	+	+		

Table 2. Monthly complete blood counts performed on WSU-D. Months represent age of the alpaca cria. NR: Not Reported

	Ref. Range	6 Mos	7 Mos	8 Mos	9 Mos	10 Mos
WBC (/µL)	8000-12000	7900	11400	3688	6603	4000
Band Neutrophils (/µL)	<200			37	66	400
Segmented Neutrophils (/µL)	4700-14900	1501	1140	443	3037	960
Lymphocytes (/µL)	700-4800	2765	4788	2102	2509	2080
Monocytes (/µL)	<1100	237	798	1033	726	560
Eosinophils (/µL)	700-4900	3318	4496	73	264	NR
Hemoglobin (g/dL)	11.9-19.4	10.4	10.6	10.1	10.3	8.1
PCV (%)	27-45	24	22	23	24	18
Plasma Protein (g/dL)	5.1-7.8	5.7	6.0	6.8	5.7	3.4
Fibrinogen (mg/dL)	100-400	300	500	NR	400	200

Table 3. Monthly complete blood counts performed on WSU-S. Months represent age of the alpaca cria.

Cells (Ref Range)	4 Mos	5 Mos	6 Mos	7 Mos	12 Mos	15 Mos
WBC 8000-12000/µL	13694	6500	8200	7300	10000	5344
Band Neutrophils <200/µL						742
Segmented Neutrophils 4700-14900/µL	6439	1820	3116	2117	6600	2491
Lymphocytes 700-4800/µL	6302	3380	4182	3796	2700	1590
Monocytes <1100/µL	959	1170	492	1022	500	424
Eosinophils 700-4900/µL			410	219	100	
Hemoglobin 11.9-19.4 g/dL	12	11.9	12.2	12.3	10.8	8.9
PCV 27-45%	29	26	27	27	25	21
Plasma Protein 5.1-7.8 g/dL	6.5	6.4	6.5	6.1	4.5	6.0
Fibrinogen 100-400 mg/dL	400	400			300	700

Table 4. Monoclonal antibodies (mAb) used to identify cell populations in whole blood collected from BVDV PI alpacas

mAb	Isotype	Specificity
DH59B1A	IgG_1	CD172a
GB45A	IgG_1	WC1 (expressed on subset of and γδ T cells)
GC50A1	IgM	CD4
LH41A	IgG_{2a}	B cells
LT3A1	IgG_1	CD5 (predicted)
LT5A	IgG_{2a}	CD8
LT10A	IgG_{2a}	CD6 (predicted)
LT97A	IgG_{2b}	αβ and γδ T cells (predicted)

Table 5. Flow cytometry results from BVDV persistently infected alpacas WSU-D and WSU-S. Months indicate age of the alpaca.

Cell Phenotype	Control	WSU-Alpaca D		WSU Alpaca S			Normal Values	
	Mean	7 Mos	9 Mos	5 Mos	7 Mos	11 Mos	Mean	Range
αβ T lymphocytes	26	64	31	25	51	29	27	11-47
CD4+	16	39	17	22	21	21	21	11-49
CD8+	11	14	12	4	25	9	10	4-28
CD4/CD8 ratio	1.5	2.8	1.4	5.5	0.8	2		1-5
CD4/CD8 dbl pos	0	11	0	2	1	0		
γδ T lymphocytes	17	6	9	7	1	9	1	4-26
B lymphocytes	53	19	42	54	21	32	4	26-60
Monocytes	4	11	16	8	31	13	19	6-39

CHAPTER 4

DISSEMINATED BOVINE VIRAL DIARRHEA VIRUS IN A PERSISTENTLY INFECTED ALPACA (VICUGNA PACOS) CRIA

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Abstract

Bovine viral diarrhea virus (BVDV) is an emerging infectious pathogen of concern to the alpaca industry. A 4-month-old, intact, male alpaca cria was diagnosed as persistently infected with BVDV based on repeated positive antemortem polymerase chain reaction (PCR) and virus isolation (VI) assays, and negative serologic titers to BVDV. Immunohistochemistry, real time reverse transcription PCR,

and VI performed on tissues collected at necropsy demonstrated disseminated BVDV-1b infection. Virus was detected in multiple tissues, including parotid salivary gland, testes, prostate, kidneys, skin, and gastrointestinal tract. Demonstration of BVDV in previously unreported tissues suggests additional potential routes of BVDV transmission in alpacas.

Introduction

A 4-month-old, intact, male alpaca (*Vicugna pacos*) cria presented to the Washington State University Veterinary Teaching Hospital (Pullman, WA) with a history of decreased weight gain, ill thrift, and anorexia. The cria was from a herd of 45 animals and was diagnosed as persistently infected (PI) with bovine viral diarrhea virus type 1 (BVDV-1), based on three repeated positive tests by polymerase chain reaction (PCR) and virus isolation (VI) on EDTA blood samples obtained at 3, 4, and 5 months of age, and negative serologic titers to BVDV on serum samples. Upon presentation, the cria was bright, alert, and responsive with normal temperature, pulse, and respiration. The cria was deemed to be significantly underweight (13.6 kg) compared to his herd mates. All other findings on physical examination were within normal limits. Complete blood cell count revealed a mild left shift (band neutrophils 309/µl; reference [ref.] range <200/µl), high normal fibrinogen (400 mg/dl; ref. range 100–400 gm/dl), anemia (PCV 21%, ref. range 27–45%), decreased hemoglobin (9.4 g/dl; ref. range 11.9–19.4 g/dl), and marked anisocytosis. Serum biochemical profile was within normal limits.

Therapy with tulathromycin (5.5 mg/kg IM) and a nutritional supplement had been initiated approximately one week earlier due to a suspected upper respiratory tract infection. Because of the grave prognosis due to PI status and general poor health of the cria, as well as financial considerations, the owners declined further antemortem diagnostics tests or treatment and elected to have the cria hospitalized for temporary monitoring. Over the next eight days, the cria was observed to be ambulating, eating, drinking, urinating, and defecating normally. Florfenicol therapy (22 mg/kg, subcutaneous, q 48 h) was initiated on day four because of a suspected respiratory infection. On the morning of day nine, the cria was found dead in its stall and transferred to the Washington Animal Disease Diagnostic Laboratory (Pullman, WA) for complete postmortem examination.

Diagnostic Testing and Results

Gross postmortem examination revealed acute, locally extensive bronchopneumonia of the cranial lung lobes; caudal lung lobes were mottled red to pink and moderately firm. Several well delineated (0.5–1.5 cm in diameter) ulcers were observed on the mucosal surface of gastric compartments 1 (C1), 2 (C2), and 3 (C3). Numerous petechiae were noted on the surface of the spleen. No other gross lesions were identified. Tissues were collected in 10% neutral buffered formalin, routinely processed, and embedded in paraffin blocks. From the paraffin blocks, 4-µm sections were cut and stained with hematoxylin and eosin. Lung, liver, and a tracheal swab submitted for aerobic culture revealed mixed bacterial growth; similar samples were negative for *Mycoplasma* culture.

Histologic examination of tissue from the cranial lung lobes revealed moderate, multifocal, suppurative bronchopneumonia. Sections from the remaining lung lobes were characterized by patchy infiltrates of minimal to low numbers of lymphocytes, plasma cells, and rare neutrophils within the interlobular and alveolar septa. Gastric compartments 1 and 2 had multifocal gastritis, characterized by a mild to moderate infiltration of the lamina propria by variable admixtures of lymphocytes, plasma cells, eosinophils, neutrophils, and macrophages. No fungi were identified. In several sections of both large and small intestine, the lamina propria was mildly to moderately expanded by neutrophils, lymphocytes, and plasma cells. The liver exhibited moderate, multifocal, random hepatocellular necrosis consistent with bacteremia. Submandibular, inguinal, and mesenteric lymph nodes had mildly to markedly reduced numbers of lymphocytes within the cortex and medulla; mesenteric lymph nodes were most severely affected. The cortex and medulla of the thymic lobules and the white pulp of the spleen had moderately to severely reduced lymphocyte numbers; the thymic corticomedullary junction was indistinct.

Immunohistochemical (IHC) detection of BVDV antigen was performed on sections of formalin-fixed, paraffin-embedded tissues using monoclonal antibody (mAb) 15c5 (IDEXX MoAB 15c-5 (anti-BVDV EO), IDEXX Laboratories, Westbrook, ME, USA) at a 1:1000 dilution in a streptavidin-biotin-immunoperoxidase technique with diaminobenzidine as a chromogen (LSAB™2 Kit, Dako North America Inc., Carpinteria, CA, USA).[36] Large amounts of BVDV antigen were detected in the following tissues: parotid salivary gland, testis, prostate, esophagus, C1, C2, C3, right kidney, bone marrow, liver, lung, spleen, thymus, and the mesenteric

and submandibular lymph nodes. In sections of parotid salivary gland, there was strong intracytoplasmic immunoreactivity within acinar epithelial cells and occasional ductular epithelial cells (Fig. 1). Similarly, a section of testis revealed frequent cells within the seminiferous tubules that had strong intracytoplasmic immunoreactivity for BVDV antigen (Fig. 2). Scattered groups of epithelial cells within the convoluted tubules of the kidney had mild to moderate intracytoplasmic immunoreactivity for BVDV, while rare macrophages within the interstitium had intracytoplasmic immunoreactivity for BVDV. Within the esophagus and C1, there was BVDV immunoreactivity within the cytoplasm of basaloid epithelial cells. Compartment 2 had immunoreactivity of both the basaloid epithelial cells within the areas of stratified squamous epithelium and within the glandular epithelium of the saccules. In C3, there were frequent areas of immunoreactivity within the cytoplasm of glandular epithelial cells and within some of the gastric ulcers. Within the lung, thymus (Fig. 3), spleen, and lymph nodes, there were rare to frequent cells (presumptive macrophages) that had intracytoplasmic immunoreactivity for BVDV antigen, while scattered Kupffer cells within the liver had similar immunoreactivity. Frequent myeloid precursor cells and presumptive macrophages within the bone marrow had similar intracytoplasmic immunoreactivity for BVDV antigen.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to detect and subtype BVDV in tissues was performed using a duplex TaqMan real-time PCR procedure as previously described.[37] Following extraction of total RNA with TRIzol (Invitrogen Corp., Carlsbad, CA, USA), BVDV-1 was detected in kidney, liver, spleen, lung, and thymus. Bovine viral diarrhea virus was isolated from the same

tissues in BVDV-free bovine turbinate cells.[22] Bovine viral diarrhea virus RNA was extracted from the supernatants of virus-infected cells using a commercial RNA extraction kit (QIAamp® Viral RNA Mini Kit, Qiagen Inc., Valencia, CA, USA) and detected via TaqMan real-time PCR procedure as previously described.[37] To obtain a longer amplicon for sequencing, a separate RT-PCR reaction was run, using recombinant *Thermus thermophilus* (rTth) DNA polymerase (Applied Biosystems, Foster City, CA, USA) to amplify a highly conserved 290-bp portion of the 5'-untranslated region (5' UTR) of the BVDV genome as previously described.[38] Polymerase chain reaction amplicons were visualized on a 1.5% agarose gel containing ethidium bromide, excised using a sterile scalpel blade under ultraviolet illumination, and purified using a nucleic acid purification kit (Bio-Rad Freeze 'N Squeeze™ Kit, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's directions. Amplicon DNA was sequenced directly on both strands by a local vendor (Amplicon Express Inc., Pullman, WA, USA) using a commercial sequencing kit (Amersham DYEnamic ET Terminator Cycle Sequencing Kit, Amersham Biosciences, Piscataway, NJ, USA) with analysis on a DNA sequencer (Applied Biosystems, Foster City, CA). Sequencing reactions were done in duplicate, and sequences were confirmed by sequencing of both strands. Forward and reverse sequences were aligned using the ClustalW algorithm.[39] Each sequence was compared to the GenBank nucleotide sequence database for similarity using BLASTn (nucleotide Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence most closely matched that

of BVDV-1b (98% sequence identity to GenBank accession # AY159530 and other type 1b accessions) when compared with sequences in GenBank.

Discussion

Bovine viral diarrhea virus is considered to be primarily an infectious pathogen in cattle, but other even-toed ungulates including camelids have been reported to be susceptible.[41] Early studies in camelids suggested either resistance or decreased ability to transmit BVDV due to low seroprevalence, minimal clinical disease, and lack of pathology after experimental infections.[24,27] However, current evidence suggests that BVDV may be an emerging and significant disease of alpacas.[23,28,29,32,34] The recent increase in cases within alpacas is likely multifactoral and may involve the emergence of a novel viral strain, differences in animal management practices, or increased awareness of BVDV by alpaca owners. The BVDV genome is known to be dynamic, with replication of any isolate giving rise to a viral swarm until a predominant strain emerges, and one of these may have an increased predilection for alpacas.[34,42] Preliminary research indicates the recent BVDV infections in alpacas have been primarily noncytopathic BVDV-1b strain. [23,28,29,32]

Research on PI bovine fetuses and calves has identified virus in all tissues and shedding occurring from multiple sites.[43,44] Previous reports on four confirmed PI alpacas identified virus in various organs but did not mention reproductive tissues or salivary gland.[28,32,34] Unlike those cases, the cria in the present report had disseminated BVDV infections which included parotid salivary gland, esophagus, C1–3, testes, prostate, and kidneys. Identification of virus in these organs indicates

potential routes for transmission through communicative and reproductive behaviors. Alpacas spit as a communication tool within the herd, and breeders frequently use the behavior to evaluate sexual receptivity of the female. Breeding involves cervical penetration by the penis and deposition of semen deep inside both uterine horns. The uterine trauma occurring during the breeding process may increase the risk of BVDV transmission.[45]

Bovine viral diarrhea virus infection in calves is also associated with "weak calf syndrome" and "shipping fever," in part due to viral immunosuppressive effects. Clinically, poor growth and recurrent infection in the animal in the present study mimicked that seen with many PI calves. Similar signs have also been reported in other PI alpacas.[28,32,34] The similarities between the disease in cattle and alpacas suggest that the viral pathogenesis may be comparable.

The fetal outcome of BVDV infection in naive, pregnant cattle is variable and dependent on the stage of gestation at which infection occurs. It is believed that cattle become persistently infected when fetal infection occurs prior to immunologic development, and viral proteins are recognized as "self antigens" and therefore tolerated.[46] The gestational exposure time for BVDV immunotolerance in alpacas has not been fully identified, but appears to occur in the first trimester. The dam of the PI cria in the present study was exposed to a suspected nonclinical PI or transiently infected alpaca while located at the breeding facility during the first 90 days of her pregnancy. It was unknown if the female was exposed prior to pregnancy or continually during this time period. The animal was not exposed to cattle during her pregnancy.

Research in the cattle industry has identified fetal infection rates of over 10%, even in vaccinated herds, with the rate of PI calves approximately 0.5%.[47] Fetal infection rates are suspected to be lower within the alpaca industry due to the lower population and the recent emergence of the virus within the species; however, because potential PI crias are typically euthanized to prevent herd exposure and limit farm reputation damage, this cannot be confirmed.

The alpaca cria in the current case had a disseminated BVDV infection, which included the reproductive, salivary, and upper gastrointestinal tract tissues. These findings suggest a significant risk for BVDV transmission due to alpaca behavior and management practices. Subclinical to acute infections have been estimated to cost the cattle industry $50 to $400 per head, for all animals in the herd, not just those suffering disease.[48] With the current value of many female alpacas beginning at approximately $10,000 per animal, the potential economic losses for alpaca producers due to BVDV are significant therefore continued testing and biosecurity efforts are warranted.

Figure 1. Parotid salivary gland with immunohistochemical staining for Bovine viral diarrhea virus (BVDV) antigen. Bovine viral diarrhea virus immunoreactivity within the cytoplasm of acinar epithelial cells and rare ductular epithelial cells.

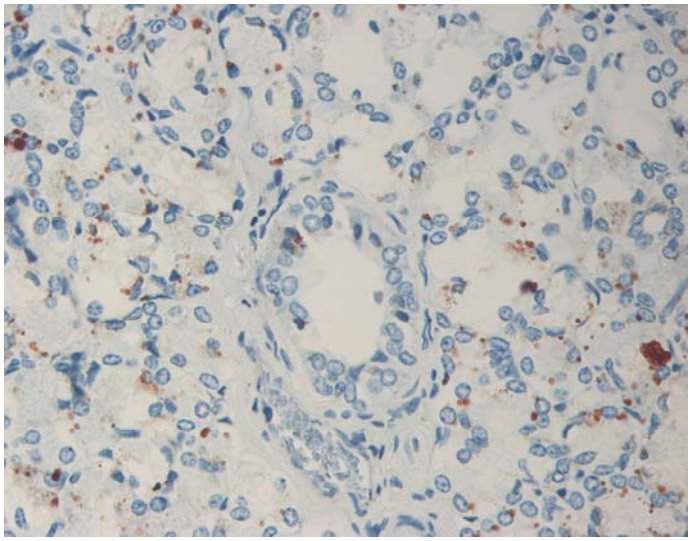


Figure 2. Immature testis with immunohistochemical staining for BVDV antigen. Bovine viral diarrhea virus immunoreactivity within the cytoplasm of cells of the seminiferous tubules.

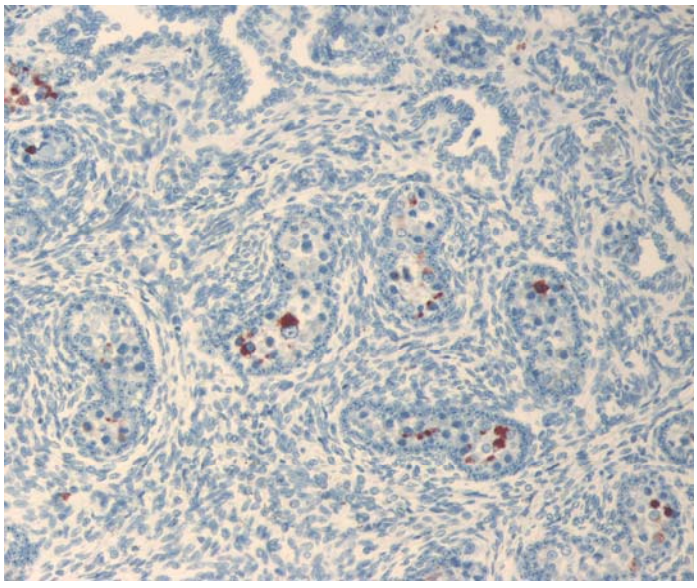
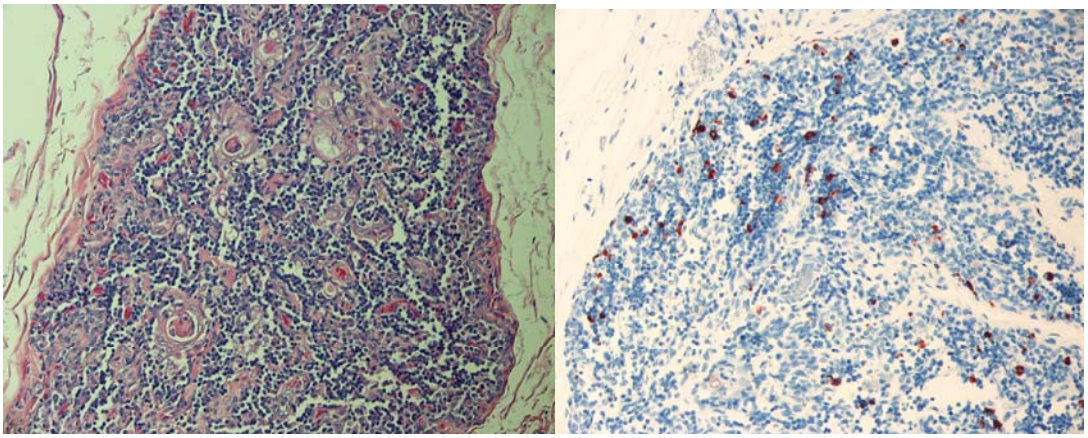


Figure 3. Hematoxylin and eosin stained section of thymus (Panel A) showing severe thymic atrophy with lymphoid depletion. Immunohistochemical staining of the thymus shows BVDV antigen within the cytoplasm of presumptive macrophages (Panel B).



Panel A Panel B

CHAPTER 5

EXPERIMENTAL BOVINE VIRAL DIARRHEA VIRUS INFECTION IN MALE ALPACAS (*VICUGNA PACOS*)

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Abstract

Bovine viral diarrhea virus (BVDV) has been reported to infect multiple animal species and determining its pathogenesis in alpacas has been of importance to the camelid industry. This research involved the experimental inoculation of five male alpacas with an alpaca isolate of BVDV (WSU 05-10966). This virus genotype (1b), was representative of other alpaca BVDV isolates. Alpacas were studied over the course of 14 days, and sampled to determine antemortem viral shedding and the location of BVDV antigen postmortem by immunohistochemistry. Clinical symptoms were mild over the 14 day observation period. There was a transient leukopenia noted

in three animals. By day 7 post inoculation, virus was obtained from the saliva of two alpacas and from the blood of all five alpacas. At necropsy, BVDV antigen was detected in the parotid salivary gland of one animal and saliva of two animals, but not in the testicular tissues or accessory sex glands. This is the first report to describe the presences of BVDV in saliva and salivary glands of alpacas suggesting an important potential route of transmission between animals.

Introduction

The pathogenesis of bovine viral diarrhea virus (BVDV) in camelids, primarily alpacas and llamas, is not completely understood. Earlier studies concentrated on serological evaluation which detected variable levels of BVDV seropositive populations.[24,27] Experimental studies have been limited due to the lack of availability of alpaca or llama field isolates of BVDV. One experimental study using a bovine strain of BVDV in pregnant llamas indicated that while the animals seroconverted, there were no ill effects noted upon fetal development and subsequent birth of crias.[27] Natural studies have expanded our knowledge regarding BVDV infection in alpacas to include the occurrence of a persistently infected (PI) state similar to what has been reported in cattle.[28,31,32,34] This study was designed to determine what clinical effects an alpaca-derived isolate of BVDV would have on naïve, healthy, male alpacas, and to determine if BVDV could be detected in selected body tissues and secretions which would account for the spread of BVDV amongst reproductively active animals.

Materials and methods

Test subjects

Five intact male alpacas (#1-5) ranging in age from 2.5 months to 48 months were selected for experimental inoculation. One male alpaca (#6), aged 48 months, was not inoculated and served as a contact control. Serum samples for BVDV serology and whole blood samples for BVDV polymerase chain reaction (PCR) were collected six days prior to experimental inoculation and every four days thereafter. All six animals were seronegative (BVDV serum neutralization antibody titer) and BVDV PCR negative at the beginning of the experiment.

Challenge virus inoculum

A pestivirus (WSU 05-10966) was initially isolated from a 12 month old alpaca that was anorexic. The alpaca was tentatively identified as being persistently infected with BVDV based upon its negative BVDV serologic status coincidental with successful isolation of BVDV from whole blood. The virus was cultured on bovine turbinate (BT) cells (BVDV free) in minimum essential media, supplemented with 10% fetal bovine serum (BVDV and BVDV antibody free), penicillin, streptomycin, gentamicin and Fungizone (JR Scientific, Inc., Woodland, CA, USA). The virus was typed as BVDV 1b by nested PCR (Figure 1). The low passage (p5) virus inoculum used to challenge the experimental animals was amplified in BT cells, and frozen at -80°C until animals were inoculated. The virus titer was determined to be $10^{6.5}TCID_{50}$/ml.

Experimental Design

Five male alpacas were inoculated by the intravenous and intrarectal routes, utilizing 10ml of BVDV inoculum per route. The intrarectal route was chosen as a convenient route for mucous membrane experimental infection. Alpacas were randomly assigned to be euthanized on days 7 and 14 post inoculation. The single in-contact control alpaca was euthanized on day 15. This study was conducted with approval, and in accordance with the Washington State University Institutional Animal Care and Use Committee. A sample size of five alpacas was chosen to limit animal use and because the effects of transient BVDV infection in camelids was unknown.

Sample collection and analysis

All six animals received initial physical examinations, and blood was obtained for a CBC, serology, virus isolation, and PCR. Saliva was collected antemortem by placing a 2 inch cotton/gauze roll in the animal's mouth for 5 minutes. The saliva was extracted using a 60cc syringe and expressed into a sterile red top tube. At necropsy, a complete set of tissues were collected and divided between fresh-frozen and fixed in 10% buffered formalin. At necropsy, seminal fluid was collected for virus isolation and PCR.

Antibodies to BVDV were determined by the serum neutralization (SN) assay, using the Singer strain of BVDV as the challenge virus. Animals were considered positive if antibody levels were equal to or greater than 1:8 dilution of the serum. Virus isolation was performed in 24-well trays pre-seeded 48 hours earlier with BT

cells. Detection of BVD by RT-PCR was conducted according to Bhudevi and Weinstock.[37] Briefly, RNA was extracted from tissues, EDTA blood, and body fluids using the QIA amp viral RNA extraction kit (Qiagen Inc., Valencia, CA, USA). Real time Taqman PCR was performed using a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA, USA) to detect and type the BVDV from the experimental animals. The sequence matched that of BVDV 1b (99% sequence identity) when compared with sequences in Genbank.

Genotyping of BVDV alpaca isolate

Genotyping of the alpaca BVDV isolate was based on phylogenic analysis of highly conserved sequences from the 5' untranslated region (UTR). Total RNA was extracted from cells infected with each isolate and a 268 nucleotide sequence from the 5' UTR was amplified and sequenced as described previously. Determination of BVDV1 subgenotypes was based on comparison of type strains for BVDV1 subgenotype identified by Vilcek et al.[6] The mean sequence homology was determined using an unpaired geometric mean analysis (UPGMA) (Higgins-Sharp algorithm/CLUSTAL4, MacDNASIS software package, Hitachi Software Engineering Co., Yokohama, Japan).

Histopathology and Immunohistochemistry

A complete compliment of tissues was collected at necropsy for histopathologic examination. Briefly, tissue samples were collected into 10% buffered formalin, processed overnight in an automatic processor (Tissue-Tek VIP, Sakura

Finetek Japan Co., LTD, Toyko, Japan), embedded in paraffin, and 4 μm thick sections were placed onto glass slides. The sections were de-parafinized with xylene and stained with hematoxylin and eosin.

Sections of fixed parotid salivary gland and reproductive tissues including testes, vas deferens, epididymis, ampulla, prostate, and bulbourethral gland were evaluated by immunohistochemistry (IHC) for evidence of BVDV antigens using the avidin-biotin-peroxidase method. Briefly, sections were deparafinized and treated with 3% H_2O_2 in methanol for 10 minutes. The samples were treated with 0.06% Pronase in Tris buffered saline (Dako North America, Inc., Carpinteria, CA, USA) for 10 minutes for antigen retrieval, and blocked with 5% normal goat serum for 10 minutes. Monoclonal antibody 15c-5 (Syracuse Bioanalytical, Inc., Syracuse, NY, USA) was diluted 1:5000 in blocker of 5% normal goat serum and incubated for 30 minutes at room temperature for antigen detection. Replicate sections were incubated with isotype-matched, irrelevant monoclonal antibody as a negative control. Primary antibody binding was detected using biotinylated goat anti-mouse IgG (Signet Laboratories, Inc., Bedham, MA, USA) for 30 minutes and labeled with Ultra Streptavidin (Signet Laboratories, Inc., Bedham, MA, USA) for 30 minutes. Dako K3464 AEC (Dak North America, Inc., Carpinteria, CA, USA) was used as the chromagen. Positive control tissue included skin from a BVDV infected bovine calf. Negative control tissues were incubated with an irrelevant isotype matched monoclonal antibody.

Results

Clinical profiles on the experimentally infected alpacas revealed that three alpacas (#2, 3, and 4) developed pyrexia (maximum of 104.8, 103, and 102.6°F respectively) at 6-7 days post inoculation (dpi). Three alpacas (#1, 4, and 5) developed neutropenia beginning 3 dpi, with alpaca #1 remaining neutropenic until euthanasia on 14 dpi. The other two animals returned to normal white blood cell counts by the next blood sampling. Alpaca #3 was lymphopenic at 7 dpi at which time it was euthanized. None of the animals developed anemia, however four alpacas (#1, 2, 3, 5) had decreasing pack cell volumes beginning at 7 dpi until euthanasia. Alpaca #5 developed transient diarrhea during days 6-9 pi. The in-contact control animal (#6) remained clinically normal during the course of the experiment

The serological and virological results indicated that three of the five experimentally infected animals seroconverted by 14 dpi (Table 1). However, the two animals that did not seroconvert were euthanized at 7 dpi and may not have had sufficient time to develop detectable BVDV antibodies. All five experimental animals were viremic by 5 dpi. Two of the five animals were positive for BVDV antigen in saliva samples at 7 dpi. The in-contact control animal remained seronegative to BVDV and no virus was detected from any salivary samples collected. Gross examination at necropsy did not revealed any abnormalities in the alpacas. Similarly, there were no histologic lesions observed, however the parotid gland on one animal was BVDV antigen positive by immunohistochemistry (IHC) (Figure 2). No virus was identified in seminal fluid collected at necropsy nor was virus detected by IHC in the

reproductive tissues. No gross or histologic lesions were observed in the control animal, and none of this alpaca's tissues were reactive to BVDV antigen by IHC.

Discussion

The results of this study indicated that following experimental inoculation of male alpacas with an alpaca-derived isolate of BVDV, there was a short-term viremia coincident with seroconversion to BVDV. There were effects noted upon blood profiles with animals displaying neutropenia, lymphopenia, and decreasing hematocrit values. Several of the alpacas developed transient fevers and one had diarrhea for three days. Virus isolation from salivary samples was positive in two of the five alpacas yet no virus was identified in seminal fluid samples.

There were no gross or histopathologic lesions in animals necropsied at 7 and 14 days post inoculation. Staining of parotid salivary gland and reproductive tissues by BVDV-specific IHC revealed that the salivary gland was positive in one of the experimentally infected animals.

The one in-contact control animal did not seroconvert during the 14 day observation period indicating that either the experimentally infected alpacas were not shedding communicable levels of BVDV, or that it took longer than the 14 day observation period to detect an immune response by the BVDV SN assay.

Bovine viral diarrhea virus was not detected in testicular tissues and seminal fluid in this group of alpacas which was in contrast to what has been reported with an experimental inoculation of BVDV into three 2-year-old bulls.[49] Although no clinical abnormalities were noted other than a spike in temperature (104-105.8°F), there was

evidence of sustained viral infection in the testes out to 7 months post infection in two of three of the bulls. Testicular biopsies were not obtained prior to this time to determine when the infection was first detectable; however semen from two bulls was found to be PCR positive at days 8 and 10 post inoculation. The differences between BVDV infection in alpaca males and young bovine bulls is of importance in understanding the epidemiology of the virus in non-bovine species. Although our study concluded at 14 days post infection, there was no evidence that the virus replicated in, or established infection in the sexual glands of the male alpacas. The lack of occurrence of BVDV in the seminal fluid and other reproductive tissues, suggests that sexual transmission of BVDV in alpacas may not be as common as other routes. It has been documented that uterine trauma occurs during the breeding process potentially exposing the female to infectious agents.[45] However, the apparent lack of BVD virus in seminal fluids could suggest a lower risk for females to acquire this disease during breeding.

The occurrence of BVDV in the salivary secretions of two of the male alpacas suggests however, that saliva may be an important route of disease transmission in alpacas. Immunohistochemistry positive BVDV staining in the parotid salivary gland indicates that viral replication was occurring, and that virus may have been amplified prior to the onset of an immune response. Alpacas commonly use “spitting” as a communication tool between animals. This trait and the presence of BVDV in saliva and salivary gland should be further evaluated to determine ease of viral transmission between animals.

Finally, conclusions drawn from a single in-contact control animal must not be overstated. The failure of this animal to seroconvert or produce detectable virus might suggest that transmission requires longer contact time or that it is not as efficient in other species. Clearly, studies involving larger populations of animals are necessary to more definitively determine the frequency and efficiency of various routes of transmission within this species.

Figure 1. Phylogenetic tree of alpaca isolate WSU 05-10966 in comparison with other pestivirus isolates. Note that the virus is placed in the BVDV 1b subgenotype together with two prototype bovine strains, TGAC and NY-1, and another alpaca isolate 023909 (see arrow).

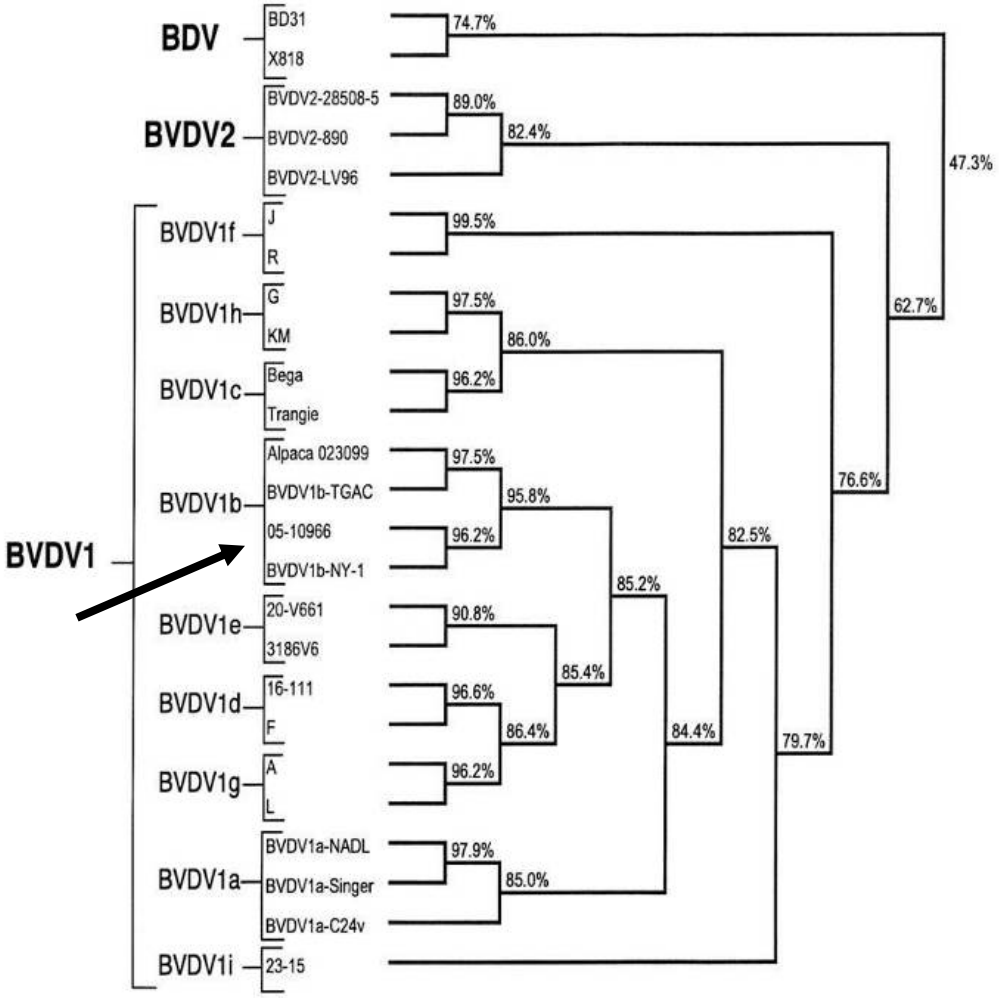

Figure 2. Immunohistochemical staining for BVDV antigen in parotid salivary gland of animal # 3 counterstained with hematoxylin (×400).

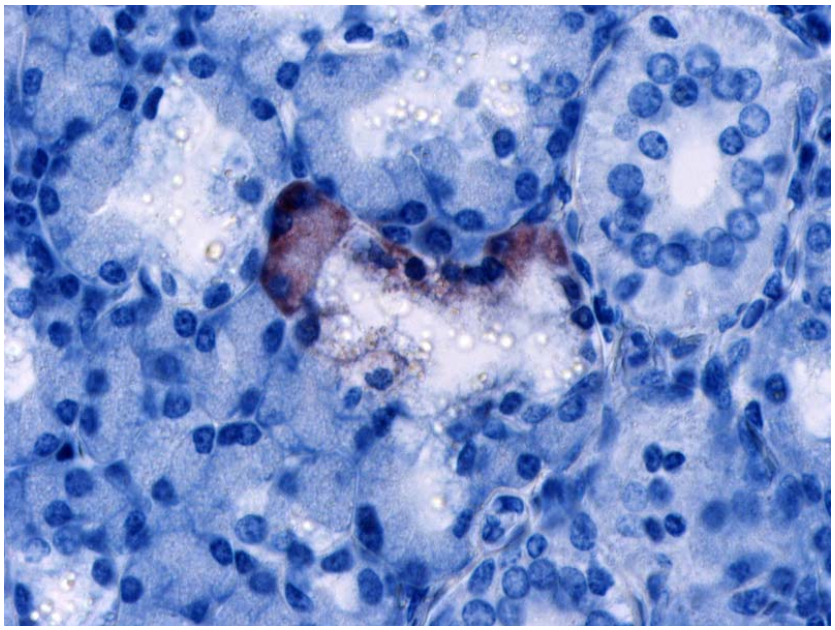

Table 1. Summary of diagnostic results from male alpacas experimentally infected with alpaca BVDV isolate (WSU 05-10966).

Animal number	Maximum BVDV antibody[a] titer post infection	BVDV isolation[b]		
		Blood	Saliva	Semen
1	1:16 (14 dpi)	+	+	-
2	<1:8 (7 dpi)	+	+	-
3	<1:8 (7 dpi)	+	-	-
4	1:32 (14 dpi)	+	-	-
5	1:64 (14 dpi)	+	-	-
6 (in-contact control)	<1:8 (15 dpe)[c]	-	-	-

[a] Serum neutralization antibodies
[b] Virus isolation in bovine turbinate cells, confirmed by PCR
[c] Days post exposure

CHAPTER 6

ACUTE BOVINE VIRAL DIARRHEA VIRUS INFECTION, TRANSMISSION, AND DISEASE EFFECTS IN ALPACAS EXPOSED TO PERSISTENTLY INFECTED ALPACAS

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Abstract

Bovine viral diarrhea virus (BVDV) reports in alpacas have been increasing; however, much is still unknown about the mechanisms of disease in this species. This report describes research performed to evaluate transmission of BVDV from

persistently infected (PI) alpacas to naïve alpacas, document shedding patterns, and determine the effects on the immunological status. Two PI alpacas were determined to shed BVDV Type 1b virus in most body fluids, and commonly available diagnostic tests were used to verify their status. The alpacas exhibited signs of ill thrift and increased susceptibility to secondary infections. Transient infections with the BVDV Type 1b strain produced only mild signs of disease in BVDV naïve alpacas. Viral shedding during the acute phase was not detected and antibody titers appeared to be protective upon re-exposure to the virus. Immunosuppressive effects were observed, which could lead to secondary infections similar to that seen in cattle.

Introduction

Bovine viral diarrhea viruses (BVDV-1 and BVDV-2) are members of the pestivirus genus that includes genetically similar viruses such as Border Disease virus of sheep and Classical Swine Fever (Hog Cholera virus) in swine.[50,51] Bovine viral diarrhea virus was first recognized as a pathogen of cattle in 1946, however BVDV does infect other even-toed ungulates with varying degrees of disease severity.[2,3] Recent reports indicate an increased number of BVDV-1b infections occurring in alpacas and describe clinical disease in species such as goats, bison, yaks, mountain goats, and white tailed deer.[2,23,28,29,31,32,34,52-56]

The recent increase in reported cases of BVDV in alpacas and other species is likely multifactoral and may involve variations in virus strain, animal management practices, as well as increased awareness and testing by producers. As with other RNA viruses, the BVDV genome is dynamic resulting from genetic mutation and gene

recombination.[57-59] It is suspected that certain BVDV strains may adapt to new hosts resulting in increased infectivity in non-bovine species such as alpacas.[1,34] Despite the increasing number of alpaca BVDV cases, much is still unknown regarding transmission, fetal infections, and clinical disease.

Research has shown that transiently (TI) and persistently infected (PI) cattle can transmit BVDV in various body fluids, with the primary portal of infection via the oronasal mucosa.[12,17,60] Transiently infected cattle typically shed BVDV for only a few days whereas PI cattle are believed to either continuously shed large quantities of virus or in some cases, inconsistently shed as evidenced by periods of intermittent viremia.[20,61-63] Both TI and PI cattle are sources of infection to other cattle. Similar shedding and transmission patterns are suspected in PI and TI alpacas, but have not been reported. Based on identification of the virus in tissues such as salivary glands, kidneys, gastrointestinal tract, lungs, testicles, and prostate, it is likely alpacas shed virus in a range of body fluids similar to cattle.[28,31,32,34]

Cattle become persistently infected with BVDV if the fetus is exposed to the virus before the immune system is fully developed, resulting in immune tolerance whereby the fetus does not recognize virus as foreign.[12] Fetal infection can occur if the dam develops a transient infection or is a PI herself.[64] Persistently infected calves typically show signs of ill-thrift and chronic illnesses; however, they can appear clinically normal.[14]

Research in BVDV PI alpacas is lacking in part due to the relative infrequency of the condition, the long gestation period of alpacas (11$\pm$ months), and the likelihood that most crias suspected of being PI's are euthanized and not reported. The present

study was designed to evaluate the serologic and viral status of alpacas suspected of being persistently infected with BVDV; to evaluate transmission of BVDV from PI alpacas to naïve alpacas; to document shedding patterns in transiently infected animals; and to determine the effects of BVDV on the immunological status of PI and TI alpacas.

Materials and Methods

Animals

Two alpaca crias from a single farm (a five-month-old intact male (WSU alpaca S) and a six-month-old female (WSU alpaca D) were donated to Washington State University Veterinary Teaching Hospital. The two crias were verified as persistently infected with BVDV based on positive BVD PCR and virus isolation (VI), and negative BVDV serology on monthly checks starting at 2-3 months of age.[22,37]

Six intact male alpacas (5 to 6-month old) were used as BVDV-negative, infection controls. The crias were verified as BVDV negative based on PCR and antibody by SN.[22,37] The cutoff for negative SN titers was 1:8. All six animals were clinically healthy.

The studies were conducted in accordance with the Washington State University Institutional Animal Care and Use Committee. The limited number of experimental animals was chosen and justified based on humanitarian grounds since BVDV is known to induce immunosuppression and increase the risk of infection with secondary pathogens in other species.

Sampling

Blood, nasal and oral swabs, and fecal samples were obtained every three days during weeks 1-3. Blood samples were then obtained weekly during the next month (weeks 4-8), then every two weeks for the next three months (weeks 9-20). Twelve milliliters of blood were aseptically obtained from the jugular vein and divided between tubes containing ethylenediaminetetraacetic acid (EDTA), acid citrate dextrose (ACD), and serum blood collection tubes (BD Vacutainer Blood Collection Tubes; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Individual nasal and oral swabs were collected from the lateral wall of the nasopharynx and buccal surface of the oral cavity. Swabs were placed in individual viral collection vials (BD Universal Viral Transport Kit (220222), Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Fecal samples were obtained by digital collection from the rectum and placed in sterile plastic specimen bags. When possible, free-catch urine samples were collected in sterile specimen cups. Samples not immediately processed were stored at -20°C until analysis.

Four months following the initial exposure period, the same six male alpacas were re-introduced to the remaining PI alpaca (WSU-S) in the same facility and pen configuration as described above. Blood, nasal and oral swabs were collected daily for one week, every third day for one week, then at one month and three months post exposure.

Virus Isolation and PCR

Whole blood and oral and nasal swabs from the PI alpacas and BVDV exposed alpacas were analyzed using virus isolation in bovine turbinate cells free of BVDV.[22] Bovine viral diarrhea virus RNA was extracted from the supernatants of virus-infected cells or from EDTA blood using the Magmax-96 Blood RNA Isolation Kit (Ambion, Inc. Austin, TX, USA). Real Time Taqman PCR to detect BVDV was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and using the ABI AgPath-ID™ BVDV Reagent Kit. The sequences were compared to the GenBank nucleotide sequence database for similarity using BLASTn (nucleotide Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Antigen ELISA

Serum samples were tested for BVD antigen by the IDEXX HerdChek Bovine Virus Diarrhea Antigen Test Kit (IDEXX Laboratories, Westbrook, ME, USA). Briefly, 100 μL of serum samples was placed in pre-wetted wells, along with positive and negative standards, and in-house reference sera. Testing was done according to IDEXX protocols. Samples with standard to positive (S/P) ratios of <0.20 were classified as "BVDV-Negative". Samples with S/P ratios of 0.20 – 0.39 were classified as "Suspect", and samples with an S/P ratio of >0.39 were classified as "BVDV-Positive". Suspect and positive reactors were assayed twice, first using the standard working detector reagent, and then using a "modified" working detector reagent to check for non-specific reactivity. Optical densities were measured using a

Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA), and calculations were made with KC-4 Bio-Tek software.

Serum Neutralization

Serum was analyzed for BVDV-specific antibodies using the SN assay as described by Evermann, et al.[22] Alpaca serum was diluted in two-fold serial dilutions using minimal essential media (MEM) plus antibiotics [2.5 μg/ml Fungizone (JR Scientific, Inc., Woodland, CA), 100 International Units (IU)/ml penicillin G, 100 IU/ml streptomycin sulfate, and 50 μg/ml gentamicin] beginning at a screening dilution of 1:4. Briefly, 0.050 ml of MEM plus was added to wells of a flat-bottom 96-well microtiter plate and 0.050 ml alpaca serum and known positive control serum were added to the appropriate wells. Serial dilutions were performed with an end volume of 0.050 ml in each well. The plate was incubated at room temperature for one hour after which 0.050 ml of BT cells (BVDV-free) were added to each well as the indicator cell type followed by incubation in a 10% humidified, 5% CO_2 incubator at 37°C. At 72 hours, the plate was read for serum neutralization of virus or cytopathic effect. The Singer strain of cytopathic BVDV was used as the challenge virus at 100-1000 $TCID_{50}$. Serum antibody titers were expressed as the reciprocal of the highest dilution of serum providing 100 percent cell protection.

Flow Cytometry

Whole blood was collected in ACD, and peripheral blood mononuclear cells (PBMC) separated by density gradient centrifugation using Accu-Paque (density,

1.086 g/ml; Accurate Chemical & Scientific Corps., Westbury, NY, USA). The buffy coat layer was collected from the interface and washed three times in phosphate buffered saline (PBS) containing 20% ACD to remove excess platelets. The cells were resuspended in PBS/ACD. Concentration of the cell suspension was determined using a hemocytometer.

One million cells were added to wells of a 96 well v-bottom microtiter plate containing two mAbs (50 µl each, 15 µg/ml) in 100 µl of dilution buffer [PBS containing 0.5% horse serum (Invitrogen/GIBCO, Gaithersburg, MD, USA), 20% ACD and 0.02% azide]. Cells were incubated for 15 min (4°C) and then washed three times in dilution buffer. Following washing, cells were incubated for 15 min (4°C) with 50 µl of isotype specific fluorescein or phycoerythrin conjugated goat anti-mouse immunoglobulin (isotype specific) antibodies (Invitrogen/Caltag, Burlingame, CA, USA). The cells were again washed two times with dilution buffer without horse serum, resuspended in 2% formaldehyde in PBS (200 µl/well) and stored refrigerated in the dark until analyzed.

An anti-pan T lymphocyte mAb was used to identify T and B lymphocytes (Table 1). T lymphocyte subsets were further differentiated using mAbs specific for CD4+, CD8+, and γδ T cells (Table 1). A FACS Calibur flow cytometer with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used to collect data. The FCS Express software (De Novo Software, Thornton, Ontario, Canada) was used to analyze data.

Experimental Exposure

Three BVDV negative alpacas (1A-1C) and the two PI alpacas (WSU-S, WSU-D) were placed together in an 8 foot by 10 foot pen (pen 1) and the other three alpacas (2D-2F) were placed in a similar sized adjacent pen (pen 2) separated by a six foot high chain link fence. Both groups were provided free choice grass and alfalfa hay, and a commercial pelleted alpaca ration with trace minerals. Alpacas in pen 1 were also provided a commercial calf starter ration due to the low body condition score of the PIs (BCS 1.5/5). Both groups had separate water sources. Pens were cleaned on a daily basis. The alpacas were housed for four weeks (weeks 1-4) after which the six non-PI alpacas were removed to a separate facility and maintained as separate groups in order to monitor serum titers. Vital parameters (temperature, pulse, and respiration rates), and compartment 1 (C1) contraction rates were obtained twice daily day on all animals during weeks 1-3 of the experiment. During the second exposure period (weeks 16 - 20) vital parameters and C1 contraction rates were obtained daily during week 16.

Results

Verification of PI status:

Throughout the duration of the project, PI status was verified on a monthly basis by PCR and serology (Table 2). Both alpacas (WSU-D and WSU-S) retained their PI status until euthanasia. Antigen ELISA was performed intermittently on both serum and ear notch skin samples and were found to be positive in both animals. Viral

shedding was verified by PCR and VI testing of periodic oral and nasal swabs. Urine samples obtained prior to euthanasia were PCR and VI positive from both PI alpaca and on feces from WSU-D.

Viral genomic sequence patterns from both PI animals most closely matched that of BVDV-1b (98% sequence identity to GenBank accession # AY159530 and other type 1b accessions) (Figure 1).

PCR and Serology

Two alpacas (1A and 1C) housed in direct contact and one alpaca (2F) housed in fence-line contact with the PI alpacas became PCR positive from EDTA blood samples on day 5; all others became PCR positive by day 8 of exposure (Table 3). All six animals remained PCR positive through day 18. Two alpacas (1A, 1C) seroconverted by day 18, three alpacas (2D, 2E, 2F) by day 21, and the final alpaca (1B) by day 27. Titers continued to increase after separation from the PI alpacas. Titers remained elevated then declined slightly around three months post exposure. Oral and nasal swabs collected from all six alpacas during the viremic stage were BVDV negative by VI and PCR testing.

During week 7, one of the PI alpacas (WSU-D) was euthanized for humane reasons. Upon re-exposure to the remaining PI alpaca (WSU-S) beginning at week 16, none of the six previously exposed alpacas produced detectable virus by PCR performed on blood samples and no significant changes occurred in serum antibody titer levels (data not shown). WSU-S was verified to be shedding BVDV prior to and throughout the second exposure period.

Complete Blood Cell Counts

Complete blood cell counts in the six non-PI alpacas revealed only mild changes. All six alpacas developed transient low-normal neutrophil counts 2-5 days after becoming viremic. Neutrophil counts remained decreased for approximately one week before returning to normal levels. None of the alpacas demonstrated increased numbers of immature neutrophils. Three to five days after becoming viremic, three alpacas (1A, 1B, 2E) developed low-normal lymphocyte counts which persisted for less than a week. Four alpacas (1B, 1C, 2D, 2E) developed mildly decreased hematocrit values (23-26%; reference range 27-45%) 3-7 days after becoming PCR positive. Mild anemia persisted for 1-3 weeks. No other abnormalities were detected.

Physical Examination

Mild changes in physical examination findings were noted (Table 4). Elevated rectal temperatures (39.3-39.6°C; reference range 37.2-39.2°C) were detected in three alpacas (1B, 1C, 2D) for 1-3 days beginning on day 11 of exposure. Alpacas in both pens developed soft stools for up to 11 days beginning on day 8 of exposure. Two alpacas (1A, 1C) housed in pen 1 developed diarrhea on days 15 and 17, respectively. Nasal discharge was present in two alpacas (2E, 2F) days 5 and 6 of exposure, though no coughing or sneezing occurred and respiratory rates remained within normal limits. Two alpacas (1B, 2D) developed transient lameness for several days, however the

cause was not identified and the lameness resolved without treatment. No abnormalities were noted during the second exposure period.

Flow cytometry

Blood from the six transiently infected alpacas, obtained four weeks prior to, and four weeks and three months after initial exposure to the PI alpacas, was analyzed by flow cytometry (Table 5). Pre-exposure lymphocyte values were within the normal range for camelids; however monocyte levels were increased in five alpacas. Pre and postexposure values were compared using a paired Student's *t*-Test (significance set at $p < 0.5$). Pre-exposure and week 4 values were significantly different for αβ T lymphocytes, B lymphocyte, monocytes, CD4+ and CD8+ T lymphocytes subsets; but not for the γδ T lymphocytes. The average percentage of T lymphocytes increased where as the percentage of B lymphocytes and monocytes were decreased four weeks following exposure to the PI alpacas. The average percentage of CD4+ and CD8+ T lymphocytes were increased four weeks post exposure as well. Samples obtained three months post exposure revealed cell distributions and percentages that were similar to the four week post exposure samples.

Discussion

Research on PI bovine calves has identified BVD virus in most tissues and viral shedding occurring from multiple sites.[43,44] Previous reports on five confirmed PI alpacas identified virus in various organs including gastrointestinal tract, testes, prostate, kidney, and parotid salivary gland.[28,31,32,34] Identification of virus in these

organs suggests potential routes for viral transmission. In the present study, viral shedding was documented from urine, nasal and oral swabs obtained from both PI alpacas and from the feces of one PI alpaca (WSU-D).

Both pens had naïve alpacas that became viremic within five days of exposure to the PI crias. The remainder became viremic before the next blood sampling three days later. Transmission of virus was suspected to be via aerosolized bodily secretions with infection likely through the oronasal mucus membrane contact since the indirect fence-line contact alpacas readily developed transient infections. Other potential exposure routes that could not be ruled out included nose to nose contact through the fence, direct contact with bodily fluids, and spitting. However, during the experiment period, manure and urination areas were not located adjacent to the shared fence line and no evidence of spitting was observed. However, during the experiment period, manure piles were not located adjacent to the shared fence line and no evidence of spitting was observed. Alpacas in pen 1 shared feed, water buckets, and a manure pile suggesting that both indirect and direct transmission was probable. Both housing situations mimicked that commonly seen on alpaca farms where either direct physical contact or indirect (adjacent, fence line) contact can occur.

After a presumed incubation period of 5-8 days, BVDV antigen was detectable by PCR for 10-13 days in previously BVDV-naïve alpacas. This finding is similar to that reported in cattle where acute BVDV infections have an incubation period of 5-7 days and viremia persists for up to 15 days.[12,14] Interestingly viral shedding was not detected in the oral or nasal secretions of the transiently infected alpacas whereas transiently infected cattle shed virus in these secretions.[65] The lack of shedding by

transiently infected alpacas may contribute to the relatively low prevalence of BVDV disease and persistent infections in alpacas.

Following the initial exposure of four weeks to the PI alpacas, the six exposed alpacas were transferred to a remote area where their BVDVantibody titers were tested every two weeks and were found to peak between weeks 9-13. At approximately week 16, the six male alpacas were re-exposed to the remaining PI alpaca (WSU-S).). Upon this secondary or re-exposure, the antibody titers did not significantly change, and in fact, appeared to be protective, as all six remained PCR negative. Again, our findings were similar to that described with acute BVDV infections in cattle wherein detectable serum antibodies were present 2-3 weeks post exposure and peaked at 2-3 months, unless exposure to PI cattle was maintained.[66,67]

Acute BVDV infections within endemic cattle herds are usually described as inapparent or subclinical. Previous reports and the results from this project, suggest a similar, mild clinical disease process in alpacas. Several animals from both pens developed mildly elevated temperatures and soft stools, and a smaller proportion developed mild diarrhea and nasal discharge. Such mild clinical signs may not be appreciated in production units unless close, daily monitoring of animals is practiced. The origin of the transient lameness that resolved in two alpacas was not discovered. However, the smooth nature of the flooring in the pens was considered a potential cause of minor trauma.

Bovine viral diarrhea virus has been demonstrated to have an immunosuppressive effect in both TI and PI cattle. Immunosuppression increases the likelihood of clinically significant secondary infections, especially when animals are

introduced to new or stressful environments such as feedlots or sale yards.[68] In cattle, BVDV affects both production and function of lymphocytes, monocytes, and neutrophils and alters immune-related cytokines involved in the immune response.[14] Transiently infected cattle typically experience lymphoid tissue depletion, leukopenia, and lymphopenia.[69,70] Some of the alpacas in this study also revealed temporary decreases in circulating neutrophil, lymphocyte, and monocyte populations. Lymphoid biopsies were not obtained, therefore the existence of lymphoid depletion could not be determined. Mild anemia was observed in the majority of transiently infected alpacas, and the condition resolved in all alpacas over time without treatment. The transient neutropenia and anemia may have resulted from temporary, viral induced, suppression of bone marrow activity.

Flow cytometric analysis of peripheral leukocyte populations revealed consistent changes in the TI alpacas after four weeks of exposure to the PI animals. Changes included increased average proportions of αβ T lymphocytes, CD4+ and CD8+ T lymphocytes, and decreased average proportions of B lymphocytes and monocytes. Decreases in B lymphocyte and monocyte populations persisted through the time of the follow up testing, three months after initial exposure to the PI animals. In contrast, acutely infected cattle have been found to have decreased proportions of CD8+ and CD4+ T lymphocytes, variable changes in B lymphocytes, and no significant variation in monocytes or γδ T lymphocytes.[70,71] In light of the sampling periods, it is possible that transient changes were not identified and the values observed may represent a rebound effect following depression of lymphocyte populations. However, the persistent depression in B lymphocyte and monocytes

populations may indicate differences in the alpaca immunological response to the virus versus that in cattle.

The poor growth and recurrent infections in the PI alpacas in the present study mimicked that seen with many PI calves and the few reports of PI alpacas.[28,31,32,34] Throughout the project, WSU-D suffered from recurrent upper respiratory tract infections which were responsive to treatment with hydrochloride (Excenel®, Pfizer Animal Health, New York, NY, USA) or florfenicol (Nuflor®, Intervet/Schering-Plough Animal Health, Summit, NJ, USA) antibiotics. Physical examinations revealed recurrent bouts of fever up to 39.8°C with signs of upper respiratory tract disease, but no anorexia, depression, or other clinical signs during these episodes. Blood work indicated a chronic process with consistently low normal leukocyte counts, neutropenia and anemia, with normal fibrinogen and globulin concentrations. Due to WSU-D's deteriorating condition, failure to respond to therapy, the alpaca was humanely euthanized at 8 months of age. Post mortem examination revealed an upper and lower respiratory tract infection with *Bordetella bronchiseptica* and *Arcanobacterium pyogenes*, together with evidence of disseminated BVDV infection.

The second PI alpaca (WSU-S), was also stunted but suffered from less frequent recurrent infections. Physical examination findings remained within normal limits and blood work revealed intermittent mild leukopenia and neutropenia similar to WSU-D. The last month prior to euthanasia, WSU-S began showing signs of anorexia with weight loss and depression, and blood work indicated an inflammatory process. Despite treatment with florfenicol (Nuflor®), the alpaca continued to decline and was humanely euthanized at 15 months of age. Post mortem examination found aspiration

pneumonia, biliary duct hyperplasia, and diffuse small intestinal villous atrophy. Bovine viral diarrhea virus antigen was only detected in the skin and urinary bladder by immunohistochemistry.

Results of the present study suggest that a likely route of BVDV infection in alpacas is via the oronasal mucosa, presumably after inhalation of viral particles present in the respiratory secretions of infected animals. The mild clinical signs observed during acute, transient infections in alpacas suggest that infections may be inapparent to owners and producers. However, the potential for immunosuppression and associated secondary infections may result in increased risks of disease for alpacas either traveling or co-mingling at various events. While the animals evaluated in this project appeared to resist subsequent infection upon secondary re-exposure to the viral-shedding, PI animals, the duration of this potentially protective effect was not determined.

Acknowledgements

The authors would like to thank Dr. Martha Delaney, Dr. Bill Davis, Ms. Sallie Bayly, Mr. John VanderSchalie, Ms. Carol Petersen, and Ms. Lorraine Tanaka for their assistance in this project.

Table 1. Monoclonal antibodies (mAb) used to identify cell populations in whole blood collected from male alpacas exposed to BVDV PI alpacas.

mAb	Isotype	Specificity
DH59B1A	IgG_1	CD172a
GB45A	IgG_1	WC1 (expressed on subset of and γδ T cells)
GC50A1	IgM	CD4
LH41A	IgG_{2a}	B cells
LT3A1	IgG_1	CD5 (predicted)
LT5A	IgG_{2a}	CD8
LT10A	IgG_{2a}	CD6 (predicted)
LT97A	IgG_{2b}	αβ and γδ T cells (predicted)

Table 2. Diagnostic results for BVDV PI alpacas [WSU-S (S) and WSU-D (D)]. The exposure project started during month 1. Diagnostic testing techniques with a (+) or (–) indicates samples were BVDV positive or negative; tests without a (+) or (-) were not run on the samples. PCR and virus isolation testing of blood samples were performed on whole blood and serum and AgELISA testing was performed on serum or ear notch skin samples. Oral and nasal samples were secretions obtained by swabbing the mucus membranes.

Month Tested	WSU Alpaca	Sample	PCR	VI	AgELISA	Serology
0	D, S	blood	+	+	+	-
1	D, S	blood	+	+	+	-
		oral & nasal	+	+		
2	D, S	blood	+			-
3	D, S	blood	+			-
		ear notch			+	
4	D	feces, urine	+	+		
	D, S	blood	+			-
5	S	blood	+		+	-
6	S	blood	+			-
		oral & nasal	+	+		
7	S	blood	+			-
8	S	blood	+			-
9	S	blood	+			-
		oral & nasal	-	-		
10	S	blood	+	+		-
		urine, oral, nasal	+	+		

Figure 1. Phylogenetic analysis and relatedness of the BVDV persistently infected alpaca crias to other known BVDV strains. Persistently infected alpacas utilized in this project are identified as WSU alpaca S (WSU-S in text) and WSU alpaca D (WSU-D).

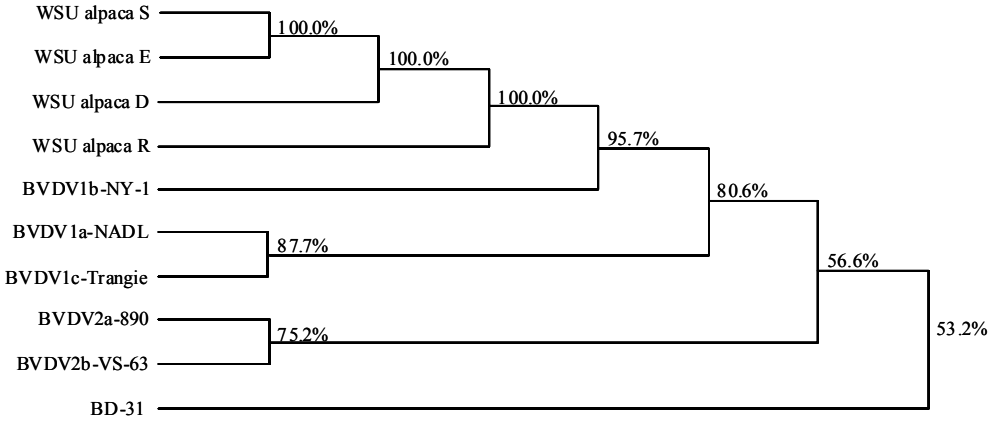

Table 3. Diagnostic results of BVDV negative males exposed to BVDV PI alpacas. Pen 1 alpacas were housed directly with the PI animals and Pen 2 provided fence line contact with the animals in Pen 1. Serology titer results represent the duration the alpacas were in contact with the PI alpacas (Days 0-28) and duration after contact was terminated (Days 63-112). Day 0 represents the start of the exposure project.

		PCR Positive	Serology Dates						
Pen	**Animal**	**Dates**	**0**	**7**	**18**	**27**	**63**	**95**	**112**
			(Housed with PI's)				(Not housed with PI's)		
1	A	Days 5-18	<4	<4	4	16	128	256	256
	B	Days 8-18	<4	<4	<4	4	256	128	64
	C	Days 5-18	<4	<4	4	32	256	512	256
2	D	Days 8-18	<4	<4	<4	64	>512	512	>512
	E	Days 8-18	<4	<4	<4	16	256	256	256
	F	Days 5-18	<4	<4	<4	8	>512	512	512

Table 4. Abnormal physical examination findings for alpacas housed with BVDV PI alpacas.

		Physical Examination		
Pen	**Animal**	**Elevated Rectal Temp °C (°F)**	**Other Findings**	
1	A	WNL	Diarrhea (days 15-17)	Soft stool in pen (days 11-19)
	B	Day 13: 39.5 (103.1)	Hind limb lameness (day 11)	
	C	Day 11: 39.5 (103.1), Day 13: 39.3 (102.8)	Diarrhea (day 17)	
2	D	Days 11-12: 39.6 (103.3)	Left hind lameness (day 13)	Soft stool in pen (days 8-19)
	E	WNL	Nasal discharge (day 6)	
	F	WNL	Nasal discharge (day 5)	

Table 5. Flow cytometry results for BVDV transiently infected alpacas. Pre-exposure sampling occurred prior to housing with the PI alpacas. Post-exposure sampling occurred during housing with the PI alpacas. Results are percent of labeled lymphocytes.

	Normal	Pre-exposure		Post-exposure	
Cell Phenotype	**Range**	**Mean +/- SD**	**Range**	**Mean +/- SD**	**Range**
αβ T Lymphocytes	11-47	17 ± 8	5-27	52 ± 5	46-58
CD4+ T Lymphocytes	11-49	17 ± 7	7-25	33 ± 5	26-38
CD8+ T Lymphocytes	4-28	5 ± 3	2-11	20 ± 4	14-25
B Lymphocytes	26-60	38 ± 10	28-55	22 ± 4	17-28
Monocytes	6-39	34 ± 19	7-56	6 ± 3	4-12

CHAPTER 7

EVALUATION OF A BOVINE VIRAL DIARRHEA VIRUS COMMERCIAL VACCINE IN ALPACAS

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Abstract

Bovine viral diarrhea virus (BVDV) is an emerging pathogen in alpacas. Eradication efforts have focused on testing and culling persistently infected alpaca crias. Vaccination as part of a control strategy was previously discouraged due to concurrent utilization of diagnostic tests for detection of infection. However, some veterinarians were administering commercial BVDV vaccinations in various alpaca herds in an attempt to reduce the economic losses from reproductive failures and persistent infections. The purpose of this research was to evaluate a commercial BVDV vaccine for safety and efficacy in alpacas. Five nonpregnant alpacas were vaccinated with a modified-live vaccine and challenged by nasal and ocular inoculation with a BVDV type 1b strain isolated from a confirmed persistently

infected alpaca. Two other nonpregnant alpacas, serving as non-vaccinate controls, were similarly challenged with the same strain. Results indicated that BVDV virus could not be detected from the vaccinated alpacas; however, unvaccinated alpacas developed signs of acute BVDV infection and virus was detected by BVDV-specific PCR. Results suggest that administration of modified-live BVDV vaccine protected the alpacas in this study from experimental challenge and no adverse effects from the vaccine were observed.

Introduction

Bovine viral diarrhea virus (BVDV) is a pestivirus that primarily causes disease in the bovine species, but it is known to infect even-toed ungulates including alpacas, small ruminants, and wild ungulates.[2,52-54,56,72] Earlier reports suggest BVDV is likely a minor pathogen that does not cause disease or persistent infections in alpacas.[24,27] However recent reports indicate an increased number of BVDV infections and persistent infections occurring within alpacas.[28,31,32,34,35,54,73] The cause for the sudden emergence of pathogenic BVDV in alpacas has not been identified, however the virus is known to be dynamic and prone to genetic modification so a strain may have emerged to which alpacas have increased susceptibility[1,34,35]

Bovine viral diarrhea virus transmission in alpacas appears to occur through separate, but interrelated vertical and horizontal modes similar to that observed in cattle. Both modes are interrelated since horizontal transmission to a pregnant alpaca or bovine can lead to vertical transmission to the fetus. Based on research in cattle, control programs including test and cull, biosecurity, and vaccination, should be

evaluated for use in alpacas to reduce or eliminate both horizontal and vertical transmission.

There are multiple testing modalities for use in cattle including various polymerase chain reaction (PCR) techniques, virus isolation (VI), antigen capture enzyme-linked immunosorbent assay (AgELISA), immunohistochemistry (IHC), and serum neutralization (SN). Sensitivity and specificity vary amongst the tests, and some are more appropriate for herd versus individual animals, and for distinguishing persistent versus transient infections. Validation for using the tests in alpacas has occurred over the past several years and the currently recommended diagnostic testing methods are PCR, VI, and SN. However PCR cannot distinguish PI from TI animals, therefore PCR positive alpacas are retested to verify PI status before recommending culling.

Biosecurity protocols can be used to prevent exposure of a herd to infected animals.[74] Research in the cattle industry has shown that factors such as transportation, change in diet, and immediate comingling increases the morbidity risk due to stress induced immune suppression and exposure to novel infectious agents.[19] A lack of biosecurity was a significant factor in the spread of BVDV through the alpaca industry in the United States.[33] Management practices included shipping juvenile and pregnant alpacas to shows and breeding facilities across the country. Herds were typically not "closed" and alpacas were brought onto farms and immediately housed with other alpacas without quarantining or testing for diseases which increased the potential for disease transmission.

Vaccination programs are the third major BVDV control mechanism in cattle, and when properly used, can significantly reduce morbidity, prevent fetal infections, and reduce reproductive losses.[7] However, during the initial outbreaks of BVDV in alpaca herds, the use of bovine-labeled, BVDV vaccines was discouraged for several reasons including the lack of safety and efficacy data, the potential for confounding diagnostic test results, and the need to validate testing strategies in alpacas. Nonetheless, the use of commercial bovine-labeled, killed BVDV vaccines occurred in an attempt by veterinarians and producers to reduce persistent infections and reproductive losses (Byers, unpublished data). Fortunately, no adverse side effects were reported from these various vaccination programs. Conversely, no beneficial effects of the vaccines were documented.

Since the cause of pathogenic BVDV emerging in alpacas has not been identified there is the risk another strain may emerge. Therefore control and potential elimination of BVDV in the alpaca species will also likely require the same multiple control modes utilized in cattle. Testing and culling PI alpacas will remain important to reduce exposure to other alpacas. Even with biosecurity methods, management factors and breeding methods maintain a higher level of exposure risk than in cattle therefore vaccination should not be discounted.

Currently, no vaccines are labeled for use in alpacas, so usage is considered "off label", and the manufacturer does not guarantee efficacy or safety. Various bovine and equine vaccines are however used in alpacas and most have only anecdotal reports of protection and side effects. The purpose of this study was to evaluate the

effectiveness of a commercial modified-live (MLV) bovine BVDV vaccine to provide protection against infection in alpacas.

Materials and Methods

Animals:

Seven clinically normal, 3-12 year-old, female alpacas were used in the study. The alpacas were test negative for BVDV by PCR on whole blood and for BVDV antibody by SN.[22,37] Five alpacas were randomly selected for vaccination and two served as non-vaccinate controls.

The study was conducted in accordance with the Washington State University Institutional Animal Care and Use Committee. The size of treatment groups was justified based on the possibility of viral-induced immunosuppression and the potential for secondary infections.

Blood and tissue sampling

Six milliliters of blood was obtained from the jugular vein and divided between ethylenediaminetetraacetic acid (EDTA) and serum blood collection tubes (BD Vacutainer Blood Collection Tubes; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Samples not immediately processed were stored at -20°C until analyzed.

Virus Isolation and PCR:

The EDTA blood samples were inoculated on to confluent cultures of bovine turbinate (BT) cells free of BVDV.[22] Bovine viral diarrhea virus RNA was extracted from the supernatants of virus-infected cells or from EDTA blood using the Magmax-96 Blood RNA Isolation Kit (Ambion, Inc. Austin, TX, USA). Real Time Taqman PCR to detect BVDV was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and using the ABI AgPath-ID™ BVDV Reagent Kit.[37]

Serum Neutralization

Serum was analyzed for BVDV-specific antibodies using the SN assay.[22] Alpaca serum was diluted in two-fold serial dilutions using minimal essential media (MEM) plus antibiotics [2.5 µg/ml Fungizone (JR Scientific, Inc., Woodland, CA), 100 International Units (IU)/ml penicillin G, 100 IU/ml streptomycin sulfate, and 50 µg/ml gentamicin] beginning at a screening dilution of 1:4. Briefly, 0.050 ml of MEM plus antibiotics was added to wells of a flat-bottom 96-well microtiter plate and 0.050 ml alpaca serum or positive control serum was added to the appropriate wells. Serial dilutions were performed with an end volume of 0.050 ml in each well. The plate was incubated at room temperature for one hour after which 0.050 ml of BT cells (BVDV-free) were added to each well as the indicator cell type followed by incubation in a 10% humidified, 5% CO_2 incubator at 37°C. At 72 hours, the plate was read for serum neutralization of virus or cytopathic effect. The Singer strain of cytopathic BVDV was used as the challenge virus at 100-1000 $TCID_{50}$. Serum antibody titers

were expressed as the reciprocal of the highest dilution of serum providing 100 percent cell protection.

Vaccination and Inoculation

Virus for inoculation was obtained from an alpaca previously determined to be persistently infected with BVDV (WSU 2007-12591). Virus was cultured on BT cells (BVDV-free) in MEM plus antibiotics with 10% fetal bovine serum (BVDV and BVDV antibody free). The virus was typed as a BVDV 1b using nested PCR and comparing to sequences to the GenBank nucleotide sequence database for similarity using BLASTn (nucleotide Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). A low passage (p5) virus inoculum was amplified in BT cells and stored at -80°C until its use in the challenge study. The viral titer of the inoculum was determined to be $10^{6.5}TCID_{50}$/ml.

Approximately four weeks prior to experimental challenge, five alpacas were transported to an isolation facility. Blood samples were obtained to confirm negative SN antibody titers, and they were vaccinated subcutaneously with 2 ml of a commercial MLV BVDV vaccine [Vista 3 SC Vaccine (Modified-live Bovine Rhinotracheitis, BVD Type 1 and Type 2), Intervet/Schering-Plough Animal Health, Millsboro, DE, USA] according to product directions. The alpacas were provided free-choice grass hay, water, and a trace mineral supplement. Three weeks after vaccination, blood samples were obtained to document the immunologic response by serum neutralization.

Immediately prior to inoculation, two unvaccinated control alpacas were transferred to the same facility and all seven alpacas were inoculated with 13 ml of BVDV. Administration involved applying drip wise approximately 6 ml of inoculum into each nostril over a 3-5 minute period, and instilling 0.5 ml of inoculum to the conjunctival membrane of each eye. None of the alpacas displayed spitting behavior or adverse respiratory signs (sneezing or snorting), though most were observed to swallow during the intranasal inoculation. It was concluded that some of the inoculum was likely administered via the pharyngeal mucous membranes or gastrointestinal tract.

Blood samples were aseptically collected from the jugular vein every 2-3 days post inoculation (pi) for four weeks, then monthly thereafter for three months. Alpacas were observed daily for clinical symptoms (lethargy, inappetence, respiratory discharge, changes in fecal consistency) for four weeks post inoculation and weekly thereafter. Vital parameters (temperature, pulse, and respiration) were only measured if clinical symptoms were detected.

Results

PCR and Serology

The vaccinated alpacas (A – E) remained BVDV PCR negative for the duration of the study while the two unvaccinated animals (F and G) were BVDV PCR positive days 6-17 pi. Serologic titers were measured prior to vaccination of alpacas A-E, and immediately prior to challenge inoculation and periodically thereafter on all seven alpacas. All vaccinated animals demonstrated negative antibody titers below

1:4 prior to vaccination and variable titers at the time of viral challenge with values ranging from 1:8 to 1:512 (Table 1). Antibody titers continued to increase after inoculation and peaked in four of five animals at >1:512. Unvaccinated alpacas G and F were antibody negative prior to challenge inoculation and seroconverted on days 23 and 50 pi, respectively. Antibody titers continued to increase until completion of sampling on day 85.

Physical Examinations

No abnormal clinical signs were observed in the five vaccinates (A-E) following vaccination or after challenge inoculation. The unvaccinated, challenged alpacas (F and G) developed serous nasal discharge and increased respiratory effort on day 15 pi. Respiratory signs and nasal discharge resolved within two weeks and no treatments were required. Vital parameters (temperature, pulse rate, respiratory rate) of alpacas F and G were monitored daily during the first week of clinical signs and remained within normal limits.

Complete Blood Cell Counts

Due to the persistent respiratory signs in the unvaccinated alpacas (F and G), a complete blood count was performed on both animals on day 23 pi. Leukocyte profiles were within normal limits, however hematocrit values were in the low normal range (28-29%; reference range 27-45%).

Discussion

The five alpacas (A-E) vaccinated with a commercial MLV BVDV vaccine appeared to be protected from challenge with a BVDV Type 1b strain when compared to unvaccinated controls. Virus was not detected by PCR in any of the vaccinated alpacas for the duration of the study. Serum neutralization titers were variable prior to inoculation and steadily increased post challenge inoculation in all five alpacas.

Bovine viral diarrhea virus was detected by PCR in the unvaccinated alpacas on days 6-17 post inoculation. This time frame is comparable to research in cattle and alpacas in which the incubation period is approximately 5-9 days with viremia persisting up to 15 days depending on virulence.[12,14] Serological titers were not detectable until day 23 and day 50 for each unvaccinated alpaca. Research in cattle indicates that an antibody response is detectable 2-3 weeks post infection and continues to rise and plateau 10-12 weeks post-infection.[66] It is possible that the delayed seroconversion of alpaca F until day 50 was a result of the immunosuppressive effects of the BVDV infection.

Acute, transient BVDV infections in cattle are typically asymptomatic or mild in nature.[13] In general, infections are manifested as a mild fever, leukopenia, anorexia, ocular or nasal discharge, oral lesions, and diarrhea, with short term viremia and shedding. Signs of increased severity can include pneumonia, secondary bacterial infections, and death.[12,14] In this study, clinical signs of disease were not observed in the vaccinated alpacas and were mild in the unvaccinated alpacas. Unvaccinated alpacas demonstrated prolonged serous nasal discharge and increased respiratory effort for up to two weeks beginning on day 15 post inoculation. During this time, the

two unvaccinated alpacas remained viremic, yet produced no detectable antibody titers. Neither anorexia nor changes in fecal consistency were observed, further indicating the mild nature of acute infection in these alpacas. Respiratory signs were attributed to either a primary effect of BVDV or BVDV-induced immunosuppression facilitating the effects of other respiratory pathogens similar to that described in the respiratory disease complex of cattle.[68]

Results of complete blood counts in the unvaccinated alpacas were unremarkable with the exception of mild decreases in hematocrit when compared to the vaccinated herdmates. One of the unvaccinated animals apparently had a true depression in hematocrit as values returned to the middle of the normal reference range by the end of the evaluation. The second unvaccinated alpaca continued to have a persistently low normal hematocrit suggesting this was the normal range for the alpaca. Since neither was clinically affected, further diagnostics to elucidate the cause of the low hematocrit, were not pursued.

A large number of commercial BVDV vaccines, both killed and MLV, are currently available for use in cattle. Newer vaccines may aid in protection against fetal infection, reproductive losses, and persistent infections. Modified live virus vaccines typically offer several benefits including the use of smaller amounts of virus, single dosing, increased immunity due to antigen replication, and increased duration of immunity.[75] Potential side effects include vaccine contamination with an unattenuated BVDV strain, vaccine induced fetal infections, short term viremia and shedding, and immunosuppression.[75,76] Since commercially available bovine respiratory vaccines contain multiple viruses in addition to BVDV, the vaccine selected for this project was

a MLV vaccine that only contained BVDV types 1 and 2 and bovine herpesvirus 1 (BHV-1, infectious bovine rhinotracheitis virus) in order to reduce the potential for confounding results. No adverse effects were seen in the vaccinated alpacas.
The single inoculation protocol used in this study was similar to that used in other BVDV vaccine studies, although a continuous exposure challenge may have been more representative of natural infections.[76-78] A similar study was planned, however the persistently infected alpaca planned for the exposure challenge succumbed to a secondary bacterial infection prior to the start of the project.

Research in cattle has identified the oronasal mucus membranes as the primary entrance for BVDV infection therefore most studies use either direct inoculation or nebulization techniques.[60] Previous work in alpacas has identified the oronasal mucus membranes as the probable route of natural infection in alpacas as well (Byers, unpublished). In this project, both nasal and ocular mucus membranes were inoculated to ensure adequate exposure to virus.

This is the first report of a challenge study evaluating the use of a commercial BVDV vaccine in alpacas. The single dose of MLV BVDV vaccine produced variable serologic titers ranging from 1:8 to >1:512. Despite this variation in titers, none of the five vaccinated alpacas developed clinical signs or produced detectable BVDV virus after challenge with live BVDV type 1. This was in contrast to the two unvaccinated alpacas that did developed minor clinical signs as well as viremia.

Results of the present study support the hypothesis that administration of a MLV BVDV vaccine containing BVD type 1 is protective against experimental challenge with BVD type 1. Because the study was conducted in non-pregnant female

alpacas, it is unknown if a MLV BVDV vaccine may be safe for use in pregnant alpacas or protective against fetal infections and reproductive losses. Furthermore the exact timing for the onset of protection after immunization has not been determined. Nonetheless, use of a MLV BVDV vaccine may be a viable option for assisting in the control of BVDV infections in alpacas. In addition to vaccination, measures to control BVDV in alpaca herds should include strategic testing, adoption of appropriate biosecurity measures, and the incorporation of specific management practices directed at minimizing risk factor associated disease.

Acknowledgement

The authors would like to thank Dr. Martha Delaney, Ms. Sallie Bayly, Mr. John VanderSchalie, Ms. Carol Petersen, and Ms. Lorraine Tanaka for their assistance in this project.

Table 1. Diagnostic testing results on alpacas vaccinated with a commercial bovine vaccine and then inoculated with BVDV Type 1b cultured from a confirmed persistently infected alpaca.

Animal	Status[a]	Serum Neutralization Antibody Titers by Day (Post Challenge Inoculation)								
		-25[b]	0[c]	8	15	23	35	50	56	85
A	Vaccinated	<4	>512	>512	>512	>512	>512	NT[d]	512	>512
B	Vaccinated	<4	64	512	>512	>512	>512	NT	>512	>512
C	Vaccinated	<4	64	128	256	256	128	NT	256	256
D	Vaccinated	<4	32	64	64	512	512	NT	>512	>512
E	Vaccinated	<4	8	32	256	256	256	NT	>512	>512
F	Unvaccinated	<4	<4	<4	<4	<4	<4	8	16	128
G	Unvaccinated	<4	<4	<4	<4	32	64	NT	128	256

[a] Alpacas A-E were vaccinated on Day -25 with Vista 3 SC Vaccine (Modified-live Bovine Rhinotracheitis, BVD Type 1 and Type 2), Intervet/Schering-Plough Animal Health, Millsboro, DE, USA.
[b] Initial BVDV antibody titers. Antibody titers were measured on all 7 alpacas prior to vaccination of alpacas A-E.
[c] Values for alpacas A-E represent post vaccination antibody titer response obtained immediately prior to BVDV challenge.
[d] NT = Not tested; indicates first time alpaca F had a detectable BVDV antibody titer.

CHAPTER 8

CONCLUSIONS

Bovine viral diarrhea virus is an emerging pathogen in alpacas. The virus is capable of causing persistent and transient infections in alpacas similar to that observed in cattle. Persistently infected alpacas appear to be capable of shedding virus in all body fluids. The PI alpaca is likely exposed to BVDV *in utero* during the first trimester with varying degrees of tissue infectivity and clinical signs. Generally, physical manifestations appear to be poor growth, ill-thrift, and fiber abnormalities, and the PI alpaca eventually succumbs to secondary illnesses.

Transiently infected alpacas generally experience acute, mild infections manifested as respiratory or gastrointestinal disease due to immune compromise and secondary illnesses. Transmission by TI alpacas appears to be limited.

Control of BVDV in alpacas will involve a multifaceted program which should include test and cull, biosecurity, and vaccination. The commonly used BVDV antemortem diagnostic tests: PCR, VI, SN, and AgELISA, have been validated for use in alpacas. Biosecurity will assist in limiting the spread of BVDV but management and breeding strategies used in the alpaca industry maintain the risk of BVDV transmission. Finally, the use of a commercial modified-live BVDV vaccine appears to be protective against acute BVDV infection of nonpregnant alpacas. However protection provided against fetal infections and reproductive losses needs to be determined.

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APPENDIX

A. VIRUS CULTURE AND ISOLATION

Primary Cultivation

1. Place the tissue(s) in a sterile Petri® dish, and snip off representative samples (size of a 25 cent coin) from each tissue using sterile forceps and scissors. Mince each sample into small pieces.
2. Add approximately 7 ml of Hood Media to a labeled stomacher bag and then add sample to be homogenized.
3. Place secured bag into the stomacher, and stomach until sufficient homogenization is achieved (120 seconds).
4. Transfer a portion (2 to 3 mls) of the homogenate into a prelabeled 5 ml sterile snap cap tube using a wide mouth disposable bulb-type pipette, and place in 4ºC refrigerator until ready to process.
5. Prepare growth media using Minimal Essential Media (MEM-Hepes), with 10% fetal bovine serum (BVDV free and BVDV-antibody free), 2.5 µg/ml Fungizone (JR Scientific, Inc., Woodland, CA), 100 IU/ml penicillin G, 100 IU/ml streptomycin sulfate, and 50 µg/ml gentamicin.
6. Seed 24-well plate with BTurb cell line and growth media. Incubate for 24-48 hours. Examine to determine if monolayer covers 80-90% of well.
7. Prepare ice bath and keep samples chilled throughout inoculation procedure. Prechill centrifuge and buckets.
8. Centrifuge sample homogenates in snap cap tubes for 15 minutes at 2500 x g at 4ºC. Transfer to ice bath immediately.

9. Using a 10 ml pipette to remove growth media from wells as needed to keep cell cultures moist. Rotate plate to keep wells moist.
10. Inoculate 0.25 ml of the centrifuged sample supernate into each of two wells of all appropriate cell lines. Inoculate the two cell control wells with 0.25 ml Hood's media.
11. Place inoculated plates in 37ºC incubator for 60 minutes (±15 minutes).
12. After incubation, pipette 1.0 ml of Hood's media into each well of all cell lines. Change pipettes between wells.
13. Return plates to 37ºC incubator.
14. Examine daily for cytotoxicity (CTE), bacterial contamination, or cytopathic effect (CPE) for up to 5 days. If CTE or bacterial contamination occurs and progresses to involve 50% of the monolayer in either or both wells, collect the cells and culture fluid, freeze at -20ºC, thaw at 37ºC, centrifuge at 2500 x g at 4ºC for 15 minutes, and re-passage supernate. [Passage 2 (P2)]. Where bacterial contamination is suspected, supernate is to be filtered through a 0.45 µl filter prior to inoculation of P2. If bacterial contamination persists, filter through a 0.22 µl filter.
15. If CPE / CTE is observed, monitor the process daily for 5 days. If not observed, see Subcultivation Procedure.

Subcultivation Procedures (Passage 2)

1. Primary cell cultures not expressing CPE at 5 days post inoculation are subpassaged to appropriate cells to ensure that no viral agents are present.

2. Collect cells and culture fluid from both wells of each sample, using a disposable bulb pipette, and transfer to sterile labeled snap cap tubes in an ice bath.
3. Freeze at -60ºC, quickly thaw in 37ºC water bath, and centrifuge at 2500 x g at 4ºC for 15 minutes. Return tubes to ice bath.
4. Inoculate 0.25 ml of sample supernate onto appropriate cell cultures and continue as for primary cultivation.
5. Samples are negative if no CPE is observed at the end of 5 days. Samples can be evaluated by PCR.

B. POLYMERASE CHAIN REACTION

Preparation

1. Oligonucleotide primers and probes used in the TaqMan assay (Positions based on the NADL genotype I strain BVD) Designation Description Sequence (5′–3′).
   - BVD Forward PCR Primer TAG CCA TGC CCT TAG TAG GAC (103-123 of the 5’ UTR)
   - BVD Reverse PCR Prime GAC GAC TAC CCT GTA CTC AGG (176-196 of the 5’ UTR)
   - BVD I Probe 6FAM- AAC AGT GGT GAG TTC GTT GGA TGG CTT - BHQ1 (145-171 of the 5’ UTR)
   - BVD II Probe 6TET- TAG CAG TGA GTC CAT TGG ATG GCC GA - BHQ1 (147-172 of the 5’ UTR)

2. Primers are ordered from Invitrogen and diluted to 40 uM in Tris EDTA (pH 8.0) buffer for storage. Working dilutions of primers are diluted to 5uM (5 pmole/µl) in PCR water (Nuclease free DEPC-treated filtered autoclaved distilled water).
3. Probes are ordered from Biosearch Technologies and diluted to 40 uM in Tris EDTA (pH 8.0) buffer for storage, and diluted to 5 uM (5 pmole/µl) in PCR water for working stocks.

RNA Extractions (Qiagen RNA extraction kits)

1. For EDTA blood, serum, tissue culture samples, extract RNA using QIAamp Viral RNA Mini-Kit.

Real-Time RT-PCR

1. Place "clean" pipettors, racks, tips, etc. into the "clean" Airclean hood, and expose to UV germicidal light for 5 to 10 minutes. Place the sample equipment into the "sample" Airclean hood, and expose to UV germicidal light for 5 to 10 minutes.
2. Prepare Master Mix for PCR, and sample addition.
    a. Remove needed reagents from freezer to thaw.
    b. In the "clean" Airclean hood, add reagents to a 2.0 ml sterile microfuge tube to make the master mix, adding PCR water first, then adding reagents in the order on the worksheet.
    c. RNase inhibitor and the enzyme mix should be kept cool, and the light sensitive probe must be kept in the dark at all times. Vortex to mix the reagents.

d. Set up Smart Cycler tubes into cooling racks which have been kept at -20ºC and pipet 17 µl of master mix into each tube.

e. Using the "sample" Airclean hood, dispense 8 µl of each extracted RNA sample into each Smart Cycler tube prepared above.

f. Fasten Smart Cycler tubes securely and briefly centrifuge for 5 seconds in the Smart Cycler microcentrifuge.

3. Run the assay:

a. Stage 1: 1 repeat @48C for 1800 sec for RT step; optics off; 95C for 600 sec to deactivate RT; optics off.

b. Stage 2: 40 repeats of 95C for 15 sec (optics off) to denature; 60C for 60 sec for annealing and extension (optics on); Cycle threshold set at 30.

C. ANTIGEN ELISA

IDEXX HerdChek Bovine Virus Diarrhea Antigen Test Kit (IDEXX Laboratories, Westbrook, ME, USA).

Sample Preparation

1. Ear notch biopsy samples.

a. Ear notches samples should be kept refrigerated except for when processing.

b. Transfer 1 square cm tissue portion into 13 ml snap-cap polypropylene tube.

c. Add 2 ml of phosphate buffered saline (IDEXX formula with ProClin biostat: 11.93g sodium phosphate dibasic, 2.21g sodium phosphate monobasic, 9.0g sodium chloride, 15uL ProClin per liter deionized water, pH to 7.4) to tube, and mix by vortexing 7-10 seconds.

d. Allow the vortexed ear notch to soak for 10 minutes at room temperature, then vortex again as before, and pour approximately 1 ml of the liquid into a ScreenMates tube for testing.

2. Warm microtiter tubes to room temperature.
3. 10X wash concentrate solution from ELISA kit should be brought to room temperature and mixed to dissolve any particles. The concentrate must be diluted with diH2O.

Test Procedure:

1. All reagents must be at room temperature and swirled to mix. Break away only as many strip wells as are needed for the samples.
2. Pre-wet wells with 200 µl of wash buffer before addition of samples. Aspirate and slap out all pre-wetting buffer before addition of samples, but do not let the wells dry out.
3. Dispense 100µL of positive and negative controls and samples into appropriate wells.
4. Cover the wells with self-adhesive film and incubate for 60 minutes at room temperature.
5. Prepare working Detector Reagent: mix 8 parts Reagent Diluent Buffer with 1 part NSB Reagent (10x concentrate) and 1 part Detector Reagent (10x concentrate).
6. Wash each well three times with about 300µL of wash buffer. Following the final aspiration, firmly tap residual wash fluid from each plate onto absorbent

material. Avoid drying between washes by leaving the plate inverted on the damp paper towel until the next reagent is dispensed into the wells.

7. Dispense 100uL of the Detector Reagent into each well.
8. Incubate for 60 minutes at room temperature.
9. Prepare working Enzyme Conjugate Reagent near the end of the incubation period (one part 10x enzyme conjugate, one part 10x NSB reagent, and eight parts reagent diluent buffer).
10. Wash each well three times with 300µL of wash buffer. Tap residual moisture out as above and avoid drying between washes.
11. Dispense 100uL of the enzyme conjugate into each well.
12. Incubate for 60 minutes at room temperature.
13. Wash each well three times with about 300µL of wash buffer as above.
14. Dispense 100µL of TMB substrate into each well.
15. Incubate for 10 minutes at room temperature in the dark for color development (blue) and then add 100µL of stop solution to each well which will change the blue color to yellow.
16. Measure and record absorbance at 450 nm through the KC-4 program.

D. SERUM NEUTRALIZATION

1. All the sera need to be heat inactivated for 30 minutes at 57°C.
2. Label one sterile bottle or tube. Keep tubes cold in an ice bath.
3. Dilute virus to working concentration based on the current virus stock and make dilutions for the back titration.

4. MEM + is prepared by combining minimal essential media (MEM) plus antibiotics (2.5 μg/ml Fungizone (JR Scientific, Inc., Woodland, CA), 100 IU/ml penicillin G, 100 IU/ml streptomycin sulfate, and 50 μg/ml gentamicin)
5. Back titration:
    a. Fill a column of a 96-well microtiter plate for the virus to be diluted, except Row A, with 0.270 ml MEM.
    b. Add 0.030 ml of each diluted virus stock to the appropriate well in Row A as labeled.
    c. Using a mutichannel pipettor, mix row A and transfer 0.030 ml from Row A and mix in Row B. Then transfer 0.030 ml from Row B into Row C and Row D, mixing each one. Take 0.030 ml from Row C into Row E, and from Row C into Row F, mixing each one. Lastly, take 0.030 ml from Row F into Row G and mix. Row H is control cells.

Testing

1. Media: Use the Matrix Impact® 12 channel electronic pipettor to add .050 ml MEM+ to all the wells of the plates.
2. Serum:
    a. Add .050 ml of the sera and positive controls to the appropriate wells of row A, using a multi-channel pipettor.
    b. Dilution: The samples will be serially diluted beginning with a 1:2 dilution in Row A, with an end volume of 0.050 ml in each well.
    c. Dilute sera with a 12 channel pipettor, or the Titertek multidiluter using the .050 ml loops and discarding .050 ml from row H.

3. Virus:
    a. Dispense the back titration first, with a Costar pipettor, into the four identical columns as marked.
    b. Pour the working virus into a sterile reservoir and drop .050 ml of the appropriate virus into each of the remaining wells of the plates with a Matrix pipettor set on the fastest speed setting, taking care not to touch the tips to any well.
    c. Tap gently to mix. Incubate at room temperature for 1 hour in the hood. Prepare bovine turbinate cells during this incubation.
4. For Bovine Turbinate (BT) cells:
    a. Cells should be between 6 and 13 days old.
    b. Make up the appropriate amount of MEM+ and set in ice water bath.
    c. Pour the growth media off the flask of BT cells.
    d. Wash with room temperature, sterile 1X CMF at least 3 times to rinse off all the media.
    e. Add 2 mls ATV (trypsin) per jumbo flask, tilt to cover the growth surface completely.
    f. Incubate at room temperature until the cells come off the bottom of the flask, about 2-5 minutes. The flasks can be tapped vigorously during this time to help shake the cells loose.
    g. Add about 10 ml MEM+ per flask to triturate the cells and wash the sides of the flask.

h. Remove all cells and MEM+ to the iced bottle and keep in an ice bath or refrigerated so they don't attach to the flask or glass bottle.

5. After 1 hour incubation of the virus, drop .050 ml of BVDV free bovine turbinate cells into each well of the plates using the Matrix pipettor. The Singer strain of cytopathic BVDV is used as the challenge virus at 100-1000 TCID50.
6. Incubation in a 10% humidified, 5% CO2 incubator at 37°C.
7. Check plates at 72 hours for serum neutralization of virus or cytopathic effect. Serum antibody titers are expressed as the reciprocal of the highest dilution of serum providing 100 percent cell protection. Dilutions are 1:4 through 1:512.