EXPERIMENTAL EXPOSURE OF NAÏVE ALPACAS TO DIFFERENT

GENOTYPES OF BOVINE VIRAL DIARRHEA VIRUS ISOLATED

FROM CATTLE AND ALPACAS

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EXPERIMENTAL EXPOSURE OF NAÏVE ALPACAS TO DIFFERENT GENOTYPES OF BOVINE VIRAL DIARRHA VIRUS ISOLATED FROM CATTLE AND ALPACAS

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Jason Wesley Johnson

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Date of Graduation

VITA

Jason Wesley Johnson, son of Eugene K. and Theresa M. Johnson, of Luverne, Alabama, was born on October 23, 1976. Reared in Luverne, Alabama, he graduated valedictorian of Crenshaw Christian Academy in 1995. He graduated from Troy State University, *cum laude*, in 1999 with a Bachelor of Science in Biology. Proceeding directly to Auburn University College of Veterinary Medicine, he graduated with Doctor of Veterinary Medicine in 2003. He married his beautiful bride Jennifer Lynn Tillery on August 9, 2003 and the couple lived in Opelika, Alabama. After a year of private mixed animal practice in Alabama, the couple moved to Greeneville, Tennessee where Jason practiced mixed animal medicine, camping, hiking, and bluegrass music. After 2 and a half years, Jason accepted a theriogenology residency position at Auburn University College of Veterinary Medicine in 2006 and entered the Graduate School to simultaneously pursue a Master of Science degree. Jennifer and Jason were blessed with a son, Elijah Gaines Johnson on May 31, 2008. Jason was awarded Diplomate status in the American College of Theriogenologists in August of 2008 and will complete his residency training and advanced degree in the summer of 2009.

THESIS ABSTRACT

EXPERIMENTAL EXPOSURE OF NAÏVE ALPACAS TO DIFFERENT GENOTYPES OF BOVINE VIRAL DIARRHEA VIRUS ISOLATED FROM CATTLE AND ALPACAS

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Master of Science, August 10, 2009
(DVM, Auburn University, 2003)
(BS, Troy State University, 1999)

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Recent outbreaks of bovine viral diarrhea virus (BVDV) have involved alpacas and evidence demonstrates that alpacas can contract and propagate the virus. The objective of this research was to compare and characterize clinical signs, viremia, nasal shedding, and seroconversion resulting from intranasal inoculation of alpacas with genotype BVDV 1b and BVDV 2 isolated from cattle and genotype 1b of alpaca origin. Three groups of six alpacas were inoculated with a different genotype of BVDV (1b [Group 1], 2 [Group 2], and 1b alpaca-strain [Group 3]). All three genotypes of BVDV induced viremia, nasal shedding and seroconversion in naïve alpacas. No clinical illness was detected in any

group. The onset of viral detection in serum was significantly different among groups; the mean onset was 4.0, 2.3, 7.5 d for Groups 1, 2, and 3 respectively. Onset and duration of viral detection in white blood cells was significantly different among groups with onset at 3.0, 2.3, and 4.7 d and cessation at 13.0, 10.0, and 12.3 d, respectively. The mean onset of viral nasal shedding was 6.9 d and was not significantly different between groups. Virus was detected post-inoculation until an average of 8.9 d in nasal secretions and 9.4 d in serum. A decrease in mean leukocyte count was observed in all three groups; however, statistically significant reductions in mean total leukocyte counts were detected only in group 1 between pre-inoculation day -7 and post-inoculation days 4 ($p=0.0003$), 6 ($p<0.0001$), and 8 ($p=0.0006$). Results demonstrate that genotype 1b and 2 strains of BVDV cause alpacas to exhibit viremia and nasal shedding of virus in a temporal pattern that is similar to the outcome of acute infection of cattle.

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I. INTRODUCTION

The *Bovine viral diarrhea virus* (BVDV) has worldwide presence and impacts its host, primarily cattle, by a vast array of reproductive, respiratory, and mucosal disease manifestations. Due to its complex pathogenesis and its economic impact on the cattle industry, the virus has been researched for over sixty years now. Advances in genetic testing in the mid-1990's phylogenetically separated members of the *Pestivirus* genus, namely BVDV into 2 genotypes, BVDV 1 and BVDV 2, and provided further capability for distinction between other Pestiviruses, such as border disease virus and classical swine fever virus (Deregt, 2005). It began to become clear that BVDV was infecting heterologous species as case reports isolated the virus and serological studies yielded evidence of exposure in pigs, sheep, deer, llamas, and most recently, alpacas (Ames, 2005; Byers et al., 2009; Carman et al., 2005; Celedon et al., 2006; Foster et al., 2005; Foster et al., 2007; Goyal et al., 2002; Kim et al., 2009; Mattson et al., 2006; Topliff et al., 2009; Wentz et al., 2003).

The appearance of the disease in alpacas was disconcerting to the cattle industry, the alpaca industry, and BVDV researchers alike. With the alpaca industry growing in the U.S.A., the animals sometimes share fence contact with cattle, especially in suburban settings. Furthermore, in general, management practices of alpacas involve interstate travel for shows and breeding. It was not known which of these aforementioned factors

were involved in dissemination of disease in alpacas (Evermann, 2006). All isolates obtained from infected alpacas were BVDV 1b (Byers et al., 2009; Carman et al., 2005; Foster et al., 2005; Foster et al., 2007; Goyal et al., 2002; Kim et al., 2009), which pointed toward an intraspecific spread of virus; however, serological studies supported interspecies spread of BVDV (Belknap et al., 2000; Mattson et al., 2006; Picton, 1993; Puntel et al., 1999; Rivera et al., 1987; Wentz et al., 2003). The existing research inolving BVDV and New World camelids had led to speculation that viremia rarely occurred in llamas exposed to the virus (Wentz et al., 2003; Mattson et al., 2006). The existence of confirmed BVDV infected alpacas seemed to assure that viremia was indeed occurring with certain strains of virus.

It was the goal of the authors to experimentally expose naïve alpacas to BVDV 1b cattle, BVDV 2 cattle, and BVDV 1b isolated from alpacas and characterize subsequent viremia, clinical signs, nasal shedding, and seroconversion. We hypothesized that exposure of post-natal naïve alpacas to BVDV would result in viremia, nasal shedding, and seroconversion.

II. LITERATURE REVIEW

Characterization of Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (BVDV) is one of the most important infectious agents in the cattle industry today and causes substantial economic losses worldwide (Grooms, 2004). Belonging to the family Flaviviridae the *Pestivirus* genus consists of the following positive-sense RNA viruses: BVDV 1, BVDV 2, classical swine fever virus, and border disease virus (Ridpath, 2005). The virus is divided based on genetic sequencing into BVDV 1 and BVDV 2 and further divided into subgenotypes (Ridpath, 2005). The virus is segregated into biotypes based upon its cytopathic effects during cell culture. Either genotype (1 or 2) may exist as a cytopthic or noncytopathic strain, with the latter biotype dominating in nature (Bolin & Grooms, 2004).

Clinical Signs of BVDV

The variety of clinical signs that BVDV induces in its host is diverse yet well documented in cattle. Basically, infections are differentiated as acute or persistent (Baker, 1995). The acutely infected animal is characterized by postnatal exposure to the virus and subsequent clearance of the virus from the body by the host's immune system

(Evermann & Barrington, 2005). In cattle, acute infections are generally subclinical but may cause anorexia, lethargy, respiratory disease, decreased milk production, mucosal lesions, and even decreased fertility and early embryonic death (Baker, 1995; Fray et al., 2000; Grooms, 2004; Kafi et al., 2002; McGowan et al., 1993; McGowan & Kirkland, 1995; Virakul et al., 1988). Cattle that are acutely infected with BVDV appear to shed low numbers of virus particles for 1 to 21 days with most shedding within a time period of 10 days or less (Thurmond, 2005). As evidenced in natural settings, this acutely infected animal may serve as a temporary source of viral transmission, but dissemination of virus predominantly occurs through persistently infected (PI) animals (Houe, 1995). A persistently infected (PI) calf develops when a fetus is exposed to a noncytopathic biotype of the virus prior to approximately 5 months of gestation. The developing fetus recognizes the viral genome as self and subsequently sheds high numbers of virus particles throughout life (Brock et al., 2005). Virus may be found in saliva, tears, nasal secretions, vaginal secretions, feces, urine, milk, and semen (Thurmond, 2005). The PI animal prevalence is between 0.5% to 2% of the cattle population (Houe, 1995). Most resources for the cattle industry are primarily focused on identifying and eliminating PI animals in order to stop the spread of disease (Grooms, 2004). Fomites have also been implicated in the spread of BVDV (Liebler-Tenorio, 2005).

Overview of Immunology of BVDV

Replication of BVDV virus begins in the nasal mucosa (the most common route of infection), spreads to the tonsils, and begins replication in the white blood cells, thereby dissiminating throughout the body (Bruschke et al., 1998; Kapil et al., 2005). In a study performed to characterize the distribution of BVDV in tissues and blood cells after acute infection with BVDV, calves were intranasally inoculated wtih noncytopathic BVDV 1b strain 890. Twelve hours after inoculation, biopsies were taken of the tonsils; intranasal swabs and blood were collected daily for detection of virus; and each day after inoculation one calf was killed in order to harvest numerous tissues for virus isolation and virus titration. Virus was isolated from the nasal mucosa 9 hours after inoculation, and could be attributed to residual from inoculum or actual viral replication in nasal mucosal tissues. The highest titers of virus on day 2 harvested tissues were found in the tonsils. Day 3 virus isolation and titer results yielded the presence of virus in the tonsils, lymph nodes, spleen and jejunum. The highest viral titer was found in the tonsils of the day 3 calves. In addition to the aforementioned tissues, the day 4 calves were found to have virus in the cecum, colon, and kidney, with viral concentrations being the highest in the ileum and Peyers patches. On day 5, all tissues except the brain were positive for virus isolation. Isolation of virus from the kidney stopped on day 5 and virus disappeared from the spleen and liver around days 8 and 9. The white blood cells remained virus isolation negative until day 4 after inoculation, when 5 out of 6 calves were positive for virus. All calves had virus present in white blood cells on days 5, 6, and 7 after inoculation. The viral titers in white blood cells were consistently lower than the titers found in the

lymphoid tissues such as the tonsil, thymus, an ileum, suggesting that the white blood cells play a role in dissemination of virus to lymphoid tissues, but high titers of viral replication actually occur in lymphoid tissues (Bruschke et al., 1998).

As previously alluded to, variations in clinical signs are determined by many factors, including viral, host, and environmental factors. Viral cellular tropism varies between strains and influences subsequent immune responses and exhibition of clinical signs (Kapil et al., 2005). Infection with BVDV can affect innate or natural immunity by decreases in function or number of neutrophils, monocytes, macrophages and dendritic cells; cytokine function may be affected as well. Adaptive responses, including cell mediated and humoral immunity are induced by BVDV infection (Kapil et al., 2005). Cell mediated immunity response to BVDV has been reported to cause decreases of 10% to 60% in lymphocyte numbers, with variation based upon viral strain (Archambault et al., 2000; Brodersen & Kelling, 1999). Humoral immune responses to BVDV causes formation of antibodies which are generally detectable 2 to 3 weeks after exposure (Kapil et al., 2005). The immunosuppressive affects of BVDV on the post-natal host have been manifested by both increasing opportunistic infections and compounding clinical signs of other pathogens. In vitro and in vivo experiments support this clinical observation by describing decreases in number and function of white blood cells (Baker & Houe, 1995). Experimental evidence to characterize immunosuppressive effects of BVDV is covered in a later section of this thesis.

BVDV and Experimental Infections in Cattle: Acute Infections

Clinical Manifestations of Experimental Acute Infections in Cattle

One of the first experimental exposures of cattle to BVDV using intra-nasal inoculation was performed by Mills and Luginbuhl in 1969, in which they exposed colostrum deprived calves to BVDV Dunkle strain of mucosal disease (Mills & Luginbugh, 1968). In this study, all inoculated calves developed diphasic fever, leucopenia, anorexia, and different levels of diarrhea. Additionally, a calf naturally exposed to the inoculated animals developed clinical signs and subsequent viremia. Clinical signs in inoculated calves recurred to varying degrees every 10 to 12 days, but seemed to decrease in severity as time from exposure increased (Mills & Luginbugh, 1968).

In another study conducted in 1981, five calves were intranasally inoculated with NADL strain of BVDV and a diphasic pyrexia, dry cough, nasal discharge, and anorexia were noted (Roth et al., 1981). Fever, hyperpnea, and anorexia 5 to 7 days post-exposure was also reported in an experimental study in which 10 cattle were intranasally inoculated with a cytopathogenic BVDV TGA (Bolin et al., 1985). TGA was an isolate that was recovered from a disease outbreak associated with high morbidity in young calves (Bolin et al., 1985). In a group of ten calves exposed to BVDV noncytopathic strain NY-1, fever developed on days 3 and 7 after inoculation. Diarrhea was noted in two animals on day 7 after exposure, which coincided with the detection of fever in the inoculated calves (Ellis et al., 1988).

A noncytopathic BVDV-TGAN isolated from an apparently healthy PI calf and a BVDV-890 isolated from a heifer that died of internal hemorrhage were intranasally inoculated separately into two groups of nonimmune calves in order to evaluate differences in virulence between these respective isolates (Bolin & Ridpath, 1992). Variation in clinical signs was noted. All nonimmune animals exposed to BVDV-890 developed fever greater than or equal to 40 ºC for a time period of 2 to 6 days during the 21 days after exposure; whereas, the nonimmune BVDV-TGAN exposed calves had fever greater than or equal to 40 ºC for a time period of 1 to 4 days during the same rectal temperature sampling period. Six nonimmune BVDV-890 challenge calves also showed yellowish diarrhea that continued for greater than 24 hours between days 7 and 12 of the 21 day post-inoculation period; all of these calves died or were euthanized during days 10 to 19 post-exposure. Two of the BVDV-TGAN challenge calves had watery brown diarrhea 4 days after inoculation, and none in this group became moribund, further highlighting the differences in clinical signs exhibited by different isolates of BVDV (Bolin & Ridpath, 1992).

In 1998, a study was conducted to compare the lesions and distribution of BVDV 2 in seronegative calves after experimental inoculation (Ellis et al., 1998). The noncytopathic Canadian 24515 isolate used for inoculation was from a fetus aborted in an outbreak of severe acute “mucosal disease-like” syndrome, respiratory disease, and death in a group of cows and calves in Ontario. Experimental calves were monitored twice daily for clinical signs, and euthanized if clinical disease became severe. By 48 hours after inoculation, all 6 calves were pyrexic, which became persistent around day 6 or 8. Severe depression and watery diarrhea appeared at this time, and all calves were

euthanized by day 12 after infection (Ellis et al., 1998). In another study, noncytopathic BVDV intranasal inoculation caused a mild nasal discharge in 13 of of 16 antibody negative calves for greater than 24 hours between 4 and 12 days after inoculation (Fredriksen et al., 1999). On day 8 after inoculation, all animals exhibited signs of anorexia and depression. Two animals had diarrhea between day 19 and 28 after exposure (Fredriksen et al., 1999). Fever was a consistent clinical finding in 8 calves exposed to BVDV 2 noncytopathic Canadian 24515 isolate (Archambault et al., 2000).

In a study conducted to compare the virulence of three different isolates of BVDV, three groups of colostrum deprived calves were intranasally exposed to BVDV 2 strain 890, BVDV 2 strain 7937, and BVDV 1 strain TGAN (Walz et al., 2001). These isolates were chosen for this challenge experiment based upon previous reports of differences in clinical signs. BVDV 890 strain, reportedly the most virulent, has been associated with more hematologic abnormalities, diarrhea, pyrexia, and even death (Bolin & Ridpath, 1990). BVDV 7937, considered the less virulent BVDV 2, has been associated with pyrexia, decreases in platelet counts, and pyrexia. Historically, BVDV 7937 was not known to induce diarrhea, leucopenia, or death in experimentally infected calves but mild disease associated with anorexia and fever was reported (Marshall et al., 1996). The BVDV 1 strain TGAN had been associated with fever but no hematologic abnormalites in experimentally infected calves (Bolin & Ridpath, 1992). Twice daily physical exams and rectal temperatures were performed for the 12 day post-inoculation period. Appetite remained normal throughout the study in all calves. All calves in BVDV 2 challenge group developed diarrhea, and only 3 of the 4 BVDV 1 challenge group calves had diarrhea. Diarrhea with BVDV 890 challenge calves had hemorrhage,

mucosal casts, and the duration was longer than for BVDV TGAN challenge calves. All BVDV inoculated calves developed a fever on days 6 through 12 after inoculation, with the longest duration of fever occurring in BVDV 890 challenge group calves (Walz et al., 2001). Clinical signs exhibited in inoculated calves were consistent with previous reports in the literature, with BVDV 890 challenge group exhibiting the most severe clinical signs when compared to the other challenge strains. The fever noted in BVDV 7937 group was consistent with previous reports, but the presence of diarrhea differed. Lastly, the BVDV TGAN challenge group had fever, which concurred with previous reports. This study yielded the conclusion that there exists a parallel relationship between the level of viremia and rectal temperature in calves experimentally infected with BVDV (Walz et al., 2001).

In 2001, a study described the clinical signs associated with experimental intranasal inoculation of naïve calves with BVDV 1d Mo1 and Mo2 cytopathic isolates (Baule et al., 2001). All inoculated calves showed respiratory symptoms; most clinical signs developed 7 days after inoculation. The most common clinical sign observed in the 21 day post-exposure period in the Mo1 challenge group was nasal discharge and intermittent fever. Nasal discharge was observed from 2 to 20 days after inoculation. One calf in this group also showed ocular discharge, coughing, and abnormal breathing; two calves had oral mucosal lesions. The Mo2 challenge group exhibited more severe clinical signs including nasal discharge, coughing, fever, abnormal breathing, oral mucosal lesions, and transient diarrhea (Baule et al., 2001).

In 2002, a study was conducted that compared the relative virulence of 5 different isolates of BVDV 2 (Kelling et al., 2002). Two of the isolates chosen were

isolated from cattle with peracute BVDV that ended in death. The other three challenge isolates chosen were considered low-virulence strains isolated from fetuses from pregnant cows who had exhibited no clinical signs other than abortion. The calves challenged with the more virulent strains showed signs of lethargy, whereas, the other challenge calves did not. Diarrhea was observed 7 to 8 days after inoculation in the calves challenged with the more virulent strains and was not seen in other calves. Severe respiratory disease was seen in the calves inoculated with the more virulent isolates of BVDV, and only mild respiratory signs were seen in the other calves. All calves inoculated with BVDV experienced a biphasic pattern of pyrexia that peaked on days 3 and 8 after inoculation. The calves inoculated with the more virulent strains of BVDV were shown to have a significantly higher rectal temperature on day 6 than low-virulence challenge calves or control calves. This study shows that experimental inoculation of the highly virulent strains of BVDV did not result in clinical signs as severe as have been observed in nature. However, the study does give evidence that variation in clinical signs between isolates in experimental challenge models correlates with relative severity of clinical signs seen between isolates in natural settings (Kelling et al., 2002).

Experimental intranasal inoculation of 8 colostrum deprived calves with a noncytopathic BVDV 2 low-virulence isolate (28508-5) was conducted in order to characterize virus tissue distribution and viral cellular replication strategies (Liebler-Tenorio et al., 2003). The isolate had been recovered from a PI calf that exhibited no signs of clinical disease. Upon challenge, no overt clinical signs of disease were observed in calves, but fever was detected on days 7 and 8 after inoculation. Under field conditions the infection of these calves would have most likely gone without detection.

Historically, BVDV 2 infections have been associated with more severe clinical disease (Archambault et al., 2000; Bolin & Ridpath, 1992; Ellis et al., 1998; Liebler-Tenorio et al., 2003), however, these challenge calves showed no clinical signs. The apparent lack of clinical signs in challenge calves is consistent with this strain's clinical manifestations in the natural host, as that animal showed no clinical signs (Liebler-Tenorio et al., 2003). Experiments performed by Ganheim et. al. in 2003 and 2005 in which calves were inoculated with a noncytopathic BVDV 1 experienced fever that began on day 7 after challenge and lasted 1 to 5 days. The fever was accompanied by varying degrees of depression (Ganheim et al., 2003; Ganheim et al., 2005). Most recently, a group of five antibody and antigen free calves were inoculated with a noncytopathic BVDV 1a, strain 456497, previously identified as a virulent field isolate. In all calves, pyrexia was detected between days 1 through 12 after challenge, with most possessing the highest fever on days 8 and 9. Some calves showed some signs of respiratory disease, such as an occasional cough, but remained normal otherwise (Collins et al.,).

Characterization of Viremias of Experimental Acute Infections in Cattle

The 1968 study in which BVDV Dunkle strain of mucosal disease was intranasally inoculated into 12 colostrum deprived calves was one of the first papers to characterize viremia after experimental respiratory exposure to virus. Two calves were determined to have virus in the bloodstream as early as 24 hours after inoculation, while the remainder of the group became viremic by 48 hours. Most calves remained viremic until days 12 or 3 post-inoculation. Beyond day 13, surviving calves were found to

possess transient periods of viremia of 24 to 48 hours. Virus was recovered in one calf up to 40 days after challenge. This study led to the conclusion that viremia occurs relatively rapidly in acute experimental respiratory exposure models with BVDV, namely with Dunkle mucosal disease virus. This study also included histopathological monitoring of virus distribution in post-exposure calves on various days. The periods of aviremia seen in calves seemed to coincide with the time frame that the virus was invading solid lymphoid tissues. The monitoring of the temporal histopathological pattern of virus distribution also gave evidence that experimental disease seemed to recur every 10 to 12 days, but decreased in clinical signs as time after challenge increased. Also, as days after challenge increased, the number of tissues from which the virus was isolated decreased (Mills & Luginbugh, 1968). A noncytopathic BVDV-TGAN isolated from an apparently healthy PI calf and a BVDV-890 isolated from a heifer that died of internal hemorrhage were intranasally inoculated separately into two groups of nonimmune calves in order to evaluate differences in virulence between these respective isolates (Bolin & Ridpath, 1992). Virus was isolated from serum, nasal secretions, lymphocytes, and platelets from the BVDV 890 challenge group 1 to 15 days after inoculation. The TGAN challenge group exhibited the presence of virus in nasal secretions, lymphocytes, and platelets on days 1 to 11 after inoculation. Virus was isolated from the serum on days 1 through 4 after challenge. Virus titration was performed on platelets and lymphocytes by performing a limiting dilution technique. BVDV 890, considered to be more virulent, was isolated from serum in challenge calves at a higher titer and for a longer period of time than for calves challenged with BVDV TGAN (Bolin & Ridpath, 1992). Further virus titration tests revealed that BVDV 890

was found in higher percentages of lymphocytes and platelets than in BVDV TGAN challenge calves. This finding led the authors to suggest that in vivo replication of BVDV 890 was greater than replication of BVDV TGAN, allowing the virus to access a wider range of cells in the body, and could be responsible for the differences seen in the severity of clinical signs between isolates. However, in challenge calves, increasing concentrations of BVDV 890 in blood and cells was not correlative with the levels of thrombocytopenia. Platelet counts decreased in both challenge groups, but the severity of thrombocytopenia was greater in BVDV 890 calves.

In a study performed to characterize the distribution of BVDV in tissues and blood cells after acute infection with BVDV, calves were intranasally inoculated wtih noncytopathic BVDV 1b strain 8900 (Bruschke et al., 1998). Twelve hours after inoculation, biopsies were taken of the tonsils; intranasal swabs and blood were collected daily for detection of virus; and each day after inoculation one calf was killed in order to harvest numerous tissues for virus isolation and virus titration. Virus was not isolated from the white blood cells on days 0 through 3, but was recovered from 5 out of 6 calves beginning on day 4. All calves were positive on days 5, 6, and 7 after inoculation (Bruschke et al., 1998).

A study was conducted in 1998 to compare the lesions and distribution of BVDV 2 in seronegative calves after experimental inoculation (Ellis et al., 1998). The noncytopathic Canadian 24515 isolate used for inoculation was recovered from an aborted fetus during an outbreak of severe acute “mucosal disease-like” syndrome, respiratory disease, and death in a group of cows and calves in Canada. Virus isolation results were positive for all 6 calves 2 days after inoculation with this virulent strain of

BVDV (Ellis et al., 1998). In another experiment, eight calves were infected intranasally with the same noncytopathic BVDV 2 Canadian 24515 isolate in order to evaluate the clinical, hematologic, and immunological results of acute BVDV infection. Virus was isolated from white blood cells from day 5 through 12 after inoculation (Archambault et al., 2000).

In a study conducted to compare the virulence of three different isolates of BVDV, three groups of colostrum deprived calves were intranasally exposed to BVDV 2 strain 890, BVDV 2 strain 7937, and BVDV 1 strain TGAN. These isolates were chosen for this challenge experiment based upon previous reports of differences in clinical signs. A limiting-dilution method, which allowed an estimation of the frequency of virus with cells, was performed on platelets and mononuclear cells. This test was run on days 0, 4, 6, 8, and 12. On day 12, the calves were euthanized and immunohistochemistry for the detection of BVDV antigen was performed on numerous tissues. Virus isolation was performed daily on white blood cells and serum. The degree of viremia was found to be significantly different among the three groups of inoculated calves, with BVDV 890 challenge group having the highest viral titer in serum, white blood cells, and platelets, followed by BVDV 7937 challenge group and TGAN challenge group, respectively. The frequency and duration of viremia in serum and white blood cells was greatest in the BVDV 890 challenge group, followed by the BVDV 7937 group and then the BVDV TGAN group. The degree of viremia was found to have a parallel relationship with rectal temperature, and an inverse relationship on blood counts. This study begs to argue that isolates known to possess a high level of viremia may cause more severe clinical signs in their hosts (Walz et al., 2001).

In 2001, a study described the clinical signs and viremia associated with experimental intranasal inoculation of naïve calves with BVDV 1d Mo1 and Mo2 cytopathic isolates (Baule et al., 2001). Virus isolation was positive in white blood cells for BVDV between days 2 and 11, and was also found more frequently in white blood cells than nasal swabs. The highest number of animals was virus isolation positive in white blood cells on day 7 after inoculation. Kelling et. al. conducted a study that compared the relative virulence of 5 different isolates of noncytopathic BVDV 2 (Kelling et al., 2002). Although clinical signs varied between groups, BVDV was isolated from all groups of experimentally exposed calves. The 2 groups of calves exposed to the more virulent field strains of BVDV were detected to have a viremia of 9 days duration, while the calves exposed to the less virulent strains of BVDV had virus in the blood for 6 to 8 days. These differences in duration of viremia correlated with the severity of clinical signs observed in calf groups, and furthermore, suggested a consistency in clinical signs of BVDV in natural settings and experimental exposures (Kelling et al., 2002).

A noncytopathic BVDV 2, 28508-5, was intranasally inoculated into eight calves in order to explore the virus-host interaction with low virulence BVDV (Liebler-Tenorio et al., 2003). After inoculation, blood was drawn on days 3, 6, 9, and 12, and a virus isolation test was performed for the presence of BVDV. Virus was found in one sample on day 3 after inoculation. By day 6, four of the six remaining calves were positive for virus; no virus was detected in day 9 or 12 samples. Most recently, a group of five antibody and antigen free calves were inoculated with a noncytopathic BVDV 1a, strain 456497, previously identified as a virulent field isolate. Virus was isolated from two calves 3 days after inoculation and isolated from all five calves 5 days after inoculation.

By 10 days after exposure, all calves were negative on virus isolation for BVDV (Collins et al.,).

Characterization of Antibody Production of Experimental Acute Infections in Cattle

Experimental intranasal exposure of 10 cattle with a cytopathogenic BVDV TGA resulted in production of BVDV antibodies 1:16 to 1:64 on day 17 after inoculation (Bolin et al., 1985). A noncytopathic BVDV-TGAN isolated from an apparently healthy PI calf and a BVDV-890 isolated from a heifer that died of internal hemorrhage were intranasally inoculated separately into two groups of nonimmune calves in order to evaluate differences in virulence between these respective isolates (Bolin & Ridpath, 1992). Both groups of calves produced virus-neutralizing antibodies to BVDV that were detected on days 10 to 13 after infection. Based upon virus isolation results, it was shown that a precipitous drop in virus associated with lymphocytes, platelets, and nasal secretions preceded antibody production. One calf maintained high levels of viremia and failed to produce antibodies by day 19 after exposure, the day of euthanasia. With all groups, neutralizing antibodies against their respective challenge virus was detected several days before the standard laboratory virus used for virus neutralization (Singer strain). In calves exposed to BVDV TGAN, virus was detected after the appearance of antibodies to their challenge strain, and through monoclonal antibody testing, was attributed to the presence of viral mutants. Mutant virus was isolated up to 15 days after inoculation. It was speculated that these viral mutants either comprised a small

population of the initial challenge virus or TGAN strain mutated in the infected calves. These viral mutants, which remained antigenically distinct, escaped virus neutralization and allowed the virus to replicate in the host for longer periods of time. Due to the replication processes employed by positive sense RNA viruses like BVDV, the presence of mutant viruses was not totally surprising, but noteworthy in that, the documentation of this phenomenon suggests that *in vivo* selection and propogation of mutant BVDV viruses could prove to be significant in the epidemiology of the virus (Bolin & Ridpath, 1992).

A study was conducted to determine the level and duration of serum antibody production in cattle exposed to BVDV (Fredriksen et al., 1999). In this study, 3 groups of 8 virus negative and seronegative calves were experimentally inoculated, either intranasally or intramuscularly, with noncytopathic BVDV isolate 93/4618-226. All calves experimentally exposed to BVDV possessed antibodies to BVDV between days 14 and 28 after inoculation. There was no statistically significant difference in antibody titer levels between experimentally infected groups, nor was there a statistically significant difference in the antibody titers between the naturally exposed group and the experimentally exposed group. All of these animals were followed for three years, and maintained high levels of virus neutralizing antibodies to BVDV for the study period. During this time, the animals remained under controlled conditions, and no new exposures to BVDV were encountered over the three year study based upon the control group in the herd remaining antibody negative. This study suggests that animals exposed to BVDV develop antibodies which remain for an extended period of time. Over the

three year time period, there was only a slight decrease in virus neutralizing antibody levels in challenge and naturally exposed calves (Fredriksen et al., 1999).

The timeline for seroconvesion in a natural exposure setting was described in a study that placed 5 seronegative calves in nose-to-nose contact with a PI calf (Niskanen et al., 2000). Three calves possessed antibodies to BVDV on day 14 after exposure, and 2 calves possessed antibodies on day 21 (Niskanen et al., 2000). In a study by Bolin et. al., experimental inoculation of calves with 3 different noncytopathic strains did not result in antibody production by day 12 after inoculation (Bolin & Ridpath, 1992). In 2001, a study interested in describing respiratory signs associated with experimental intranasal inoculation of naïve calves with BVDV 1d Mo1 and Mo2 cytopathic isolates was conducted (Baule et al., 2001). Seroconversion was detected on day 15 after exposure to BVDV 1d Mo1, and by day 17 in the BVDV Mo2 challenge group. All infected animals had the presence of BVDV antibodies in serum by 21 days after exposure (Baule et al., 2001). Experimental exposure of 12 naïve calves to BVDV resulted in seroconversion by day 19 after intranasal exposure (Ganheim et al., 2003). A group of five antibody and antigen free calves were inoculated with a noncytopathic BVDV 1a, strain 456497, previously identified as a virulent field isolate (Collins et al.,). All calves exposed to virus had seroconverted by day 21 after challenge. Antibody levels were followed for around 130 days. Antibody titres to BVDV were shown to increase in all animals at least up to 105 days after exposure (Collins et al.,).

Characterization of Nasal Shedding of BVDV of Experimental Acute Infections in Cattle

The ability of the transiently infected animal to transmit infective virions to a naïve host and induce viremia has been reported (Mills & Luginbugh, 1968). A naïve calf was placed in a pen with 2 calves that were experimentally exposed to BVDV MD by intranasal inoculation. Virus was recovered from the blood of this exposed calf 5 days after introduction. This exposed calf also developed clinical signs similar to the experimentally induced calves (Mills & Luginbugh, 1968).

In another study, a group of five calves were placed in nose-to-nose contact with a clinically healthy PI calf; all calves contracted the virus based upon clinical signs of fever, leucopenia, and subsequent seroconversion to BVDV. Fourteen naïve calves were exposed to these transiently infected calves on days 4, 7, 14, 21, 28, 35, and 42 post-exposure, and none of the fourteen showed signs of illness or produced antibodies to BVDV. Transmission from acutely infected calves to naïve cohorts was unsuccessful in this study (Niskanen et al., 2000).

Evidence does exist for the isolation of virus in nasal secretions of acutely infected animals. For example, a noncytopathic BVDV-TGAN isolated from an apparently healthy PI calf and a BVDV-890 isolated from a heifer that died of internal hemorrhage were intranasally inoculated separately into two groups of naïve calves in order to evaluate differences in virulence between these respective isolates (Bolin & Ridpath, 1992). Nasal swabs for virus isolation were collected for 21 days after inoculation. Nasal secretions were virus isolation positive from the BVDV-890 challenge

calves for 1 to 15 days following exposure; nasal secretions were positive for BVDV-TGAN challenge group for 1 to 11 days after exposure (Bolin & Ridpath, 1992). In another study that highlights the importance of the transiently infected animal, ten non-immune calves were intranasally inoculated with noncytopathic BVDV 1b strain 890 in a study performed to characterize the distribution of BVDV in tissues and blood cells after acute infection with BVDV (Bruschke et al., 1998). One animal was necropsied on each day of the 10 day study. All animals except one were found to shed infective virus for at least one sampling day. Virus isolation was positive in one animal for days 2 through 9, pointing toward the potential importance of the transiently infected animal in the epidemiology of BVDV (Bruschke et al., 1998).

Cytopathic BVDV has been studied in nasal shedding experiments. Cytopathic isolates BVDV 1d Mo1 and Mo2 were experimentally inoculated in naïve calves in order to characterize respiratory signs induced by the virus (Baule et al., 2001). Both the Mo1 isolate and Mo2 isolate inoculated calves were found to be virus isolation positive between days 2 and 11 after infection. Virus was detected in the nasal swab samples of the Mo1 challenge group fewer times than the Mo2 group, and the Mo1 group displayed less severe clinical signs of disease. Furthermore, two of the Mo2 challenge calves were found to shed virus in the nasal mucosa 21 and 31 days after inoculation (Baule et al., 2001).

Characterization of Hematologic Alterations of Experimental Acute Infections in Cattle

One of the first experiments employing intranasal exposure of BVDV to naïve calves was conducted with BVDV MD Dunkle strain (Mills & Luginbugh, 1968). This study concentrated its efforts on characterizing isolation of virus, but also mentioned hematologic parameters in infected calves. A leukopenia was detected in all inoculated calves 2 to 6 days after inoculation. By day 8 leukocyte concentrations had returned to normal ranges (Mills & Luginbugh, 1968).

Another study was conducted in which 5 cattle were experimentally inoculated via intranasal route with a noncytopathic BVDV strain NADL; this study was monitoring numerous leukocyte function parameters. Total leukocyte counts and differentials were also performed on the 25 days after inoculation (Roth et al., 1981). All animals exposed to BVDV exhibited a marked decrease in total white blood cell counts (WBC), and in the cellular subpopulations of neutrophils, lymphocytes, and eosinophils. The nadir of the mean WBC count occurred 4 days after inoculation which reflected around a 50% drop in total WBC count when compared to pre-inoculation WBC counts (Roth et al., 1981).

Cytopathic BVDV inoculated intravenously into 10 calves caused a decrease in the total WBC count of 35% by day 4 after inoculation when compared to pre-exposure values (Bolin et al., 1985). Lymphocytes were examined in this study, and viral exposure caused a decrease in total numbers of both B and T lymphocytes, and in the percentage of T lymphocytes. Neutrophils had recovered to preexposure levels by 7 days after inoculation, but lymphocyte numbers remained lower. Day 17 samples reflected a

recovery in all percentages and absolute numbers of WBCs to their pre-inoculation values (Bolin et al., 1985).

Transient leucopenia was also reported in a group of calves intranasally exposed to noncytopathic BVDV NY-1 (Ellis et al., 1988). The leucopenia was detected on days 3, 5, and 7 after inoculation and was approaching or below normal limits for cattle. Neutropenia and decreases in lymphocytes was detected on the same sample days. Total WBC counts were approaching normal limits by 9 days after challenge (Ellis et al., 1988).

A noncytopathic BVDV-TGAN isolated from an apparently healthy PI calf and a BVDV-890 isolated from a heifer that died of internal hemorrhage were intranasally inoculated separately into two groups of nonimmune calves in order to evaluate differences in virulence between these respective isolates (Bolin & Ridpath, 1992). Blood was drawn for hematolgic analysis on days 0, 2, 5-14, and 21 after exposure. The only hematologic abnormality discovered in the BVDV-TGAN group was the incidence of lymphopenia in one calf, whereas, all calves exposed to BVDV-890 were found to possess hematologic abnormalities comprised of leucopenia, lymphopenia, neutropenia, and thrombocytopenia. The composition of hematologic abnormalities seen in the BVDV-890 challenge group varied between calves, as each calf did not possess all abnormalities (Bolin & Ridpath, 1992). Other noncytopathic isolates such as BVDV 7937 and BVDV 126 have been found to equally induce reduction in platelet counts in experimentally challenged, naïve calves (Marshall et al., 1996).

Noncytopathic BVDV 2 Canadian 24515 was intranasally inoculated into eight seronegative calves in order to examine clinical and immune responses (Archambault et

al., 2000). All calves inoculated with BVDV exhibited a significant drop when compared to controls in leukocyte counts by day 3 to 5 after infection, and cell counts remained depressed through day 12, which was the termination of the study. Decreases in neutrophils, lymphocytes, and monocytes all contributed to the leucopenia seen in this study. Additionally, all calves exposed to virus showed a significant drop in platelets when compared to control calves on day 10 and 12 after inoculation (Archambault et al., 2000).

Evidence also exists for experimentally directed natural acute infections to cause hematologic abnormalities. Six naïve calves developed significant leucopenia and lymphopenia on days 4 and 11 after exposure to a PI calf, and also exhibited a significant decrease in platelet count 4 days after exposure (Traven et al., 1991). In another study in which naïve calves were exposed to a BVDV 1 PI animal, mean leucocyte counts were found to be decreased 7 days after infection (Niskanen et al., 2000). The drop in WBC count was attributed to decreases in lymphocyte and neutrophil numbers. Testing fourteen days after infection showed that leuckocyte numbers had increased, but had not yet reached pre-exposure levels (Niskanen et al., 2000).

A study was conducted to compare the virulence of three noncytopathic strains: BVDV 2 strain 890, BVDV 2 strain 7937, and BVDV 1 strain TGAN (Walz et al., 2001). Three groups of colostrum deprived calves were intranasally exposed to these isolates and hemotology was performed for the next 12 days. Significant differences in total WBC counts were noted between control group and all BVDV exposed groups. When compared to controls, BVDV 890 challenge calves were found to have significant differences in WBC counts on post-inoculation sample days 3, 5, 7, 10, and 12. BVDV

7937 induced a significant decrease in challenge calves' total WBC counts on post-inoculation days 3 and 12 when compared to control calves; BVDV TGAN challenge calves were found to possess a significant difference when compared to control calves only on day 3 after inoculation only. Challenge of calves to BVDV 890 induced the greatest reduction in platelet counts between groups. Manual platelet counts on days 7 through 12 after inoculation revealed significant decreases in platelet numbers in all BVDV inoculated calves when compared to controls (Walz et al., 2001).

Another study was conducted that compared the relative virulence of 5 different isolates of noncytopathic BVDV 2 (Kelling et al., 2002). When compared to controls, all groups of calves intranasally inoculated with virus were found to have reduced mean total WBC counts on days 4 through 7 after exposure. The animals challenged with the 2 more virulent field isolates (BVDV 23025 and BVDV 17853) were found to possess a significant lymphopenia on days 3, 5, and 6 as compared to other BVDV challenge calves. There were no significant reductions in platelet counts detected among challenge groups. These experimental infections did not lead to clinical signs as severe as reported in naturally occurring infections of the same strain, but did provide evidence that there exists a correlative relationship between laboratory induced virulence and naturally occurring clinical signs between strains (Kelling et al., 2002).

There have been other studies that investigated the hematologic alterations induced by BVDV 2 of low virulence. Eight colostrum deprived calves received intranasal nebulization of noncytopathic BVDV 2 strain 28508-5 (Liebler-Tenorio et al., 2003). Peripheral lymphocyte numbers decreased by an average of 33% by day 3 after inoculation and 38% by 6 days after inocualation. Platelet numbers remained within the

clinically normal range for all calves. This study also included the examination of histological distribution of BVDV in multiple organs throughout the body. Two calves were euthanized on sample days 3, 6, 9 and 13 for this purpose. Histology revealed the presence of virus in numerous lymphoid tissues and intestinal mucosa during the study period. The distribution of viral antigen suggests that a BVDV 2 of low virulence did not remain restricted to nasal mucosa, but rather, spread throughout the body, even though there were relatively few clinical signs observed in challenge calves. Furthermore, the author pointed out that the mechanics of this BVDV 2 infection, historically known as more severe, are similar to experimental BVDV 1 exposures (Liebler-Tenorio et al., 2003).

Field strains reported to possess high virulence have been used in experimental models to investigate hematologic abnormalities (Ellis et al., 1998). The noncytopathic BVDV 2 Canadian isolate 24515 was isolated via a fetus form a herd in Canada that experienced a severe " mucosal disease-like" outbreak with significant mortality. When this isolate was intranasally inoculated into 6 naïve calves there was a drop in WBC counts by 50% of pre-inoculation values within 2 to 3 days. Suppression of WBC counts beginning on day 7 or 8 was accompanied by neutropenia, thrombycytopenia, lymphopenia, and anemia (Ellis et al., 1998). A noncytopathic BVDV 1 isolated from a PI calf was experimentally inoculated into 6 calves and caused a significant drop in leukocyte count when compared to controls (Ganheim et al., 2005). Daily leukocyte counts first detected the decrease in WBC counts on 3 days after inoculation and it remained low for around 4 days. Neutrophil counts dropped significantly compared to control animals, with the initial decrease detected on day 3 after inoculation; counts

remained low until 8 days after inoculation. Lymphocyte numbers decreased significantly compared to control animals, beginning on day 3 after inoculation and remained lower than pre-inoculation levels for the remainder of the 23 day study. Monocyte numbers did not vary significantly between control and BVDV inoculated groups.

Characterization of Immunosuppresive Properties of Experimental Acute Infections in Cattle

Besides the leukopenias already described, there are other distinct immunosuppressive properties that BVDV acute infections appear to exert on its host. BVDV possesses an affinity for cells of the immune system (Bruschke et al., 1998). A study conducted in 1973 investigated the in vitro effects of BVDV impairment of lymphocyte function (Muscoplat C.C. et al., 1973). Lymphocytes in cell culture exhibit a predictable proliferative response to phytohemagglutinin (PHA). Lymphocytes obtained from normal cows were cultured and lymphocyte response to PHA was compared between cultures with and without the addition of BVDV. Lymphocyte response was delayed in BVDV lymphocyte models. This led the authors to conclude that BVDV alters or inhibits certain lymphocyte metabolic function. Virus was also irradiated to prevent replication, but still allow binding and penetration of cells. Lymphocyte populations with irradiated virus did not exhibit delayed response to PHA, suggesting that the virus exerts its metabolic inhibitory affects by interference with intracellular functions (Muscoplat C.C. et al., 1973).

Similar functional defects exerted by BVDV have been described in polymorphonulear (PMN) leukocytes as well (Roth et al., 1981). After intranasal experimental inoculation with noncytopathic BVDV NADL, four parameters were used to evaluate PMN function. All 4 testing parameters suggested that BVDV induces a defect in PMN function (Roth et al., 1981).

Immunosuppresive effects of cytopathic BVDV on lymphocytes in vivo have been reported as well; intravenous viral exposure of calves caused a decrease in total numbers of both B and T lymphocytes by day 4 after inoculation and a decrease in the percentage of T lymphocytes (Bolin et al., 1985). Absolute numbers of B and T lymphocytes remained depressed by 26% and 34% of their pre-exposure value up to 7 days after exposure (Bolin et al., 1985).

A noncytopathic BVDV 1 isolated from a PI calf was experimentally inoculated into 6 calves and caused a significant drop in leukocyte count when compared to controls (Ganheim et al., 2005). Analyzation of lymphocyte subpopulations revealed a depression of T-lymphocyte numbers in BVDV challenged calves, as B-cell numbers were not affected. These BVDV infected calves were inoculated intratracheally with Mannheimia haemolytica (Mh) 4 days after BVDV challenge in order to investigate clinical outcomes of combined disease. Control calves were monitored as well, as were Mh only calves, and clinical outcomes were recorded. Bacterial challenge with Mh produced more severe clinical signs in BVDV challenged calves than control or Mh only challenge calves. The negative clinical outcome was attributed to a decreased immune response secondary to BVDV lymphocyte number depression (Ganheim et al., 2005).

BVDV and Host Range

Originally, pestiviruses were categorized by the species of animal infected; however, in recent years it has become clear that BVDV can infect multiple species other than cattle (Ames, 2005). Bovine viral diarrhea virus has been isolated in natural settings from pigs (Terpstra & Wensvoort, 1988; Paton & Done, 1994), sheep (Carlsson, 1991; Paton & Done, 1994), kids and lambs (Nettleton, 1990), deer (Frolich, 1995; Frolich & Hofmann, 1995; Nettleton et al., 1980; Van Campen et al., 2001), old-world camelids (Hegazy et al., 1996), llamas (Belknap et al., 2000; Motha & Tham, 1992), and alpacas (Byers et al., 2009; Carman et al., 2005; Celedon et al., 2006; Foster et al., 2005; Foster et al., 2007; Goyal et al., 2002; Mattson et al., 2006; Topliff et al., 2009).

A persistently infected pig was reported to have lived until slaughter at 24 months; this pig never produced antibodies to BVDV (Terpstra & Wensvoort, 1991). A littermate of this PI pig was found to have antibodies to BVDV (Terpstra & Wensvoort, 1991). Swine litters have been found to be positive for the presence of BVDV (Terpstra & Wensvoort, 1991). A group of pregnant sheep housed in close quarters with cattle were found to have contracted BVDV based on virus isolation (Carlsson, 1991). A cytopathic BVDV was isolated from two seronegative free-ranging roe deer in Germany (Frolich & Hofmann, 1995; Fischer et al., 1998). Free ranging mule deer in the United States have been found to be positive for BVDV virus isolation on numerous tissues (Van Campen et al., 2001). A hunter harvested white-tail deer was found to be positive for immunohistochemical staining for the presence of BVDV (Passler et al., 2008). BVDV

was isolated in an outbreak of reproductive disease in dromedary camels in Egypt (Hegazy et al., 1996). Abortions, stillbirths, weakness, and neonatal death occurred in calves of nine pregnant females. Gross lesions in affected animals included hemorrhages of the lymph nodes, thymus, lungs, brains and intestines. One of the weak calves had mild cerebellar hypoplasia. Histopathologic examination of tissues included lymphoid depletion and vasculitis. Cytopathic BVDV was isolated from the spleen and lymph nodes of some of the affected calves (Hegazy et al., 1996). Two captive mountain goats were BVDV positive on numerous post-mortem tissues (Nelson et al., 2008). It has been proposed that close proximity and population dynamics could allow interspecies transmission of a pestivirus (Nettleton, 1990).

Serological evidence exists for heterologous infection. A serological survey of slaughtered boars and sows revealed that 20 percent of the animals possessed antibodies to BVDV (Terpstra & Wensvoort, 1991). A survey in Norway revealed that 4.5% of sheep and 2.2% of pigs possessed high antibody titers to BVDV (Loken et al., 1991b) and Graham discovered a 30.4% seroprevalence to BVDV in sheep herds in Ireland (Graham et al., 2001). Goats in Norway were found to have a 3.6% prevalence rates for neutralizing antibodies to BVDV (Loken et al., 1991b). In Namibia, Africa, a serological study was conducted after clinical outbreaks of BVDV on several cattle farms (Depner et al., 1991). BVDV antibodies were detected in sheep and goat sera; furthermore, numerous wildlife species, such as the kudu, eland, and giraffe, were found to be antibody positive for BVDV (Depner et al., 1991). A two year serological study in Germany found numerous species of cervids including fallow deer, red deer, and roe deer to be antibody positive for BVDV (Frolich, 1995). Free living cervids were found have a

significantly higher incidence of antibodies to BVDV when compared to captive cervids (Frolich, 1995). In the United States, a serological survey conducted on mule deer revealed a 60% prevalence for serum neutralizing antibody titers to BVDV (Van Campen et al., 2001). Two out of 165 hunter harvested white-tail deer possessed antibodies to BVDV (Passler et al., 2008). A survey was conducted to detect the incidence of BVDV antibodies in zoos in the United States (Doyle & Heuschele, 1983). Antibodies to BVDV were found in 9.1% of all animals tested, and in 4.3% of unvaccinated captive exotic ruminants (Doyle & Heuschele, 1983). Bison in North America were found to possess a 31% seroprevalence for BVDV antibodies (Taylor et al., 1997).

Experimental Exposure of Heterologous Species to BVDV

Experimental exposure and infection of BVDV has been characterized in many non-bovine species, including pigs (Walz et al., 1999), sheep (Scherer et al., 2001), elk (Tessaro et al., 1999), deer (Van Campen et al., 1997; Passler et al., 2007) and llamas (Wentz et al., 2003).

Experimental Exposure of Swine to BVDV

Experimental exposure of swine to BVDV has resulted in the birth of PI animals, transient viremia, and the production of antibodies to BVDV (Table 1). Five groups of 4 antibody free pregnant gilts were exposed to four different strains (NADL, P, NY-1, 76-11610) of noncytopathic BVDV in order to determine if intrauterine infection occurs

(Stewart et al., 1980). In the 14 day sampling period after intranasal inoculation, gilts in all groups maintained a normal rectal temperature in the absence of any clinical signs. Infection did occur in all gilts, based upon the presence of virus in serum on day 7 after inoculation, or subsequent seroconversions. The majority of gilts were positive on virus isolation by 7 days after exposure. By day 21, every gilt except one had antibodies to BVDV, and this gilt seroconverted at a later sampling day. The fact that pigs became viremic, seroconverted, and virus was recovered from the fetuses of one infected gilt, confirmed the ability of BVDV to establish intrauterine infections in pregnant pigs (Stewart et al., 1980).

A study was performed to compare clinical and virologic findings induced in pigs by BVDV 1 and BVDV 2 (Walz et al., 1999). Noncytopathic isolate BVDV 1 MSU-AHDL 1330478-1 was isolated from a dairy farm in Minnesota and BVDV 2 strain 890, which, in previous studies has been associated with severe thrombocytopenia, was isolated from the gastrointestinal tract of a heifer. For this study, the challenge inocula were prepared to vary in viral titer. Thirty-two pigs were divided into 4 groups of 4 pigs each. Both groups of pigs entering into the BVDV 1 and BVDV 2 studies were then intranasally inoculated with virus. Group A was the control group; Group B was challenged with 10^3 $CCID_{50}$ virus (cell culture infectious doses); Group C was challenged with 10^5 $CCID_{50}$ virus; and Group D was challenged with 10^7 $CCID_{50}$ virus. Appetite, rectal temperatures, and fecal consistencies all remained normal in all pigs in both BVDV 1 and BVDV 2 challenge studies. Virus was isolated from the serum and WBC from groups of pigs receiving the higher doses (10^5 and 10^7 $TCID_{50}$) of the BVDV 1 isolate. Post-inoculation serum samples were positive in 1 pig in Group C on day 5 and 3 pigs on

day 7; WBC were positive in 2 pigs on day 7. From group D, one pig was positive on virus isolation in serum on day 3 after inoculation, 2 pigs were positive on day 5, and 3 pigs were positive on day 7. In this group, virus isolation revealed the presence of virus in WBC samples in 2 pigs on day 5, and 3 pigs on day 7. Virus was not isolated on any sample days from the pigs inoculated with BVDV 2 isolate. Pigs were euthanized on day 7 in order to characterize gross and histopathologic lesions and test for the presence of BVDV. BVDV 1 challenge Groups C and D were positive for virus isolation, and BVDV 2 challenge Group D was positive for virus isolation. Seroconversion was not detected in any group of pigs by 7 days after virus challenge. Based upon this study, it was concluded that BVDV 1 is able to establish viremia and infection in pigs at lower experimental doses than BVDV 2. Furthermore, the severity of clinical signs and the concentrations of viremia achieved in calves inoculated with BVDV 890 (Bolin & Ridpath, 1992) were not seen in pigs inoculated with the same strain (Walz et al., 1999).

In another study BVDV 2 was documented to have caused leucopenia and thrombocytopenia in inoculated naïve pigs (Makoschey et al., 2002). All ten inoculated pigs were positive for virus isolation in WBC. A few animals developed a slight leucopenia and or thrompbocytopenia. There were no clinical signs observed in any inoculated animals, although slight elevations in rectal temperatures were noted (Makoschey et al., 2002). In another study, pregnant gilts were intranasally inoculated with a BVDV 1 MSU-AHDL 1330478-1 in order to assess the ability of transplacental infections in pigs (Walz et al., 2004). None of the 4 gilts exhibited any signs of disease during the study period. All gilts were virus isolation positive in WBC and serum for BVDV on days 5 and 7 after inoculation, and all gilts seroconverted by 21 days after

exposure. Transplacental infection rates were poor, as only one fetus out of 43 was virus positive in the spleen (Walz et al., 2004).

An interesting study was conducted to investigate the transmission potential of BVDV among experimentally induced acutely infected pigs and the subsequent cross-protective immunity BVDV infection might provide to classical swine fever virus (Wieringa-Jelsma et al., 2006). Ten eight week old pigs, negative for BVDV on virus isolation and virus neutralization, were intranasally inoculated with a BVDV 1b strain St. Oedenrode. This noncytopathic isolate, possessing a 94.9% homology with BVDV Osloss strain, was recovered from the tonsils of a naturally infected pig and was passaged in bovine embryonic tracheal cells. Twenty-four hours after incoulation, ten more BVDV naïve pigs were placed in contact with the acutely infected weaners. Oropharyngeal fluid, serum, and leukocytes were tested by virus isolation for the presence of BVDV in both groups (intranasal challenge and contact animals) on increasing day intervals. Daily clinical signs were recorded and serology was performed to detect presence of antibodies to BVDV. Neither the BVDV inoculated group or contact pig group exhibited any clinical signs of disease. BVDV was isolated from the serum and oropharyngeal samples of the BVDV challenge during the post-exposure period. Virus was not detected in white blood cells of either group. Interestingly, one pig in the contact pig group was positive for virus isolation in oropharyngeal fluid samples on day 11 and day 14. This same animal was also positive for BVDV on virus isolation of serum on day 14 after exposure. All BVDV inoculated pigs seroconverted at day 21 after infection and one of the contact animals seroconverted by day 28 after exposure. This study indicates that BVDV can be transmitted by experimentally acutely infected pigs to naïve cohorts, although it appears

to be done so inefficiently. Lack of isolation of virus from leukocytes could potentially be attributed to lysis of porcine leucocytes in EDTA blood, and it was speculated that buffy coat isolation as previously described (Walz et al., 2004) would be more successful in future experiments to isolate buffy coats (Wieringa-Jelsma et al., 2006).

Table 1: Summary of Characterizations of Experimental Exposure Studies of Swine to BVDV

Author/year	Clinical signs	Viremia	Nasal Shedding	Hematology	Ab	Route of inoculation	Comments
Stewart/1980	none	majority by day 7 serum	N/A	N/A	21	IN	virus recovered from fetuses of 1 gilt
Walz/1999	none	earliest by day 3d; most by 7, WBC & serum	N/A	N/A	N/A	IN	inoculums varied in titer; sacrificed day 7; virus positive
Makoschey/ 2002	none	positive WBC	N/A	leucopenia; thrombocytopenia	N/A	IN	increased temp noted
Walz/2004	none	day 5-7 WBC & serum	N/A	N/A	21	IN	1 of 43 fetuses: virus + spleen
Wieringa/2006	none	positive serum and oral	contact pig contracted BVDV	N/A	21	IN	naïve contact animals + VI oral swab and serum and seroconverted

Ab= antibodies; N/A= not applicable; Y= yes; IM= intramuscular; IN= intranasal; PI= persistently infected; VI= virus isolation

Experimental Exposure of Goats to BVDV

Experimental infections of sheep and goats with BVDV have been documented. Newborn goat kids that received an intramuscular injection of noncytopathic BVDV were virus isolation positive in multiple organs 10 days after injection, giving evidence of active infection (Loken et al., 1990). Virus was not recovered from tissues from any animals 20 days or later after challenge. All kids were negative for antibodies 10 and 14

days after exposure, but positive by 20 days (Loken et al., 1990). Injection of orf vaccine contaminated with pestivirus caused abortions, weak kids, and reproductive failures in breeding goat herds (Loken et al., 1991a). BVDV was isolated from serum samples from 2 weak kids and 2 apparantly healthy kids in this outbreak. Later testing revealed the prescence of antibodies to BVDV. Sheep that had contact with the vaccinated goats also experienced weak lambs and poor-doing lambs, but BVDV was never isolated from any ante or postmortem tissues. However, serological testing revealed the presence of BVDV antibodies in these flocks. In a controlled study by the same author, naïve animals were injected with the contaminated orf vaccine and all injected were found to have antibodies to BVDV four weeks after challenge (Loken et al., 1991a).

Transmission of BVDV to naïve post-natal goats by housing with PI animals has been reported (Broaddus et al., 2007). Ten healthy goats showed no signs of clinical illness when housed with 4 PI animals, 1 of BVDV 1b and 3 of BVDV 2a. Seroconversion was detected in all goats by day 42 after exposure (Broaddus et al., 2007). A study was conducted in which twenty-four seronegative pregnant goats were housed with 3 persistently infected heifers with BVDV 2a (Broaddus et al., 2009). Half of the does aborted fetuses, with 19 of the 29 abortuses being found virus isolation positive for BVDV. Blood samples and nasal swabs to be tested for BVDV were collected and pooled from exposed does on days 6, 7, and 8 after exposure, and then every 3 weeks thereafter. All serum and nasal swab samples were negative for BVDV, but all the does seroconverted by day 42 post exposure (Broaddus et al., 2009).

Table 2: Summary of Characterizations of Experimental Exposure Studies of Goats to BVDV							
Author/year	**Clinical signs**	**Viremia**	**Nasal Shedding**	**Hematology**	**Ab**	**Route of exposure**	**Comments**
Loken/1990	none	N/A	N/A	N/A	20	IM	sacrificed day 10: VI positive multiple organs Sacrificed day 20: (-)
Loken/1991	none	serum	Contact sheep became Ab +	N/A	Y	IM	challenged goats had abortions, weak kids
Broaddus/2007	none	N/A	N/A	N/A	42	PI	
Broaddus/2009	none	not detected	not detected	N/A	42	PI	50% exposed does aborted: 19 of 29 fetuses positive on VI

Ab= antibodies; N/A= not applicable; Y= yes; IM= intramuscular; IN= intranasal; PI= persistently infected; VI= virus isolation

Experimental Exposure of Sheep to BVDV

Evidence of viremias occurring in experimental infections of sheep with BVDV exist. Pregnant ewes have been experimentally intranasally inoculated with a cytopathic BVDV in order to asses the ability of the virus to penetrate the transplacental barrier (HewickerTrautwein & Trautwein, 1994). Fetuses harvested from ewes between days 10 and 14 days after inoculation were positive for BVDV antigen in the brain. This study confirms that viremia occurs in ewes inoculated with BVDV Indiana isolate (HewickerTrautwein & Trautwein, 1994).

In another study, six different noncytopathic BVDV isolates were separately inoculated into groups of pregnant ewes (Bruschke et al., 1996). None of the ewes exhibited any clinical signs or fever during the study period. Ewes were euthanized at 2 and 4 weeks after exposure and virus was isolated from almost all fetal organs in the six

challenge groups, with the exception of one group in which no virus was found. This study also compared the distribution of viral antigen in fetuses in experimentally inoculated ewes and ewes exposed to a PI calf. Both groups contracted BVDV and viral presence and distribution in fetuses was found to be similar. Even though post-mortem virus isolation testing provides evidence that the ewes contracted virus, the ewes in contact with the PI were never found to be virus positive on nasal swab samples. Measured titers of the PI calf and the experimental inoculation were similar in titer concentration, with $10^{6.5}$ and 10^{5}, respectively (Bruschke et al., 1996). A group of ewes exposed to Australian isolate of noncytopathic BVDV 1 also contracted BVDV transplacentally (Swasdipan et al., 2001). Samples of reproductive tracts and fetuses were taken around 4 days after inoculation and virus isolation was performed. All nine ewes were positive for virus isolation on reproductive tissues, but only 2 were viremic at the time of surgery 4 days after inoculation (Swasdipan et al., 2001).

A study was conducted in which ewes at four different ranges of gestation were experimentally inoculated with noncytopathic BVDV 2, which was isolated from an acute outbreak in Brazil (Scherer et al., 2001). Less than a third of the animals experienced a transient rise in temperature or mild nasal disharge. Appetite and mentation remained normal in all ewes throughout the study. Virus isolation was preformed on buffy coat preparations collected every 2 days from inoculated ewes and BVDV was isolated from 12 of the 19 ewes during the 12 day post-exposure period. Only 2 ewes were positive for 4 consecutive samples. Serum sampling revealed the presence of BVDV antibodies as early as day 15, with all ewes seroconverting by day 30 after inoculation. Efficient transplacental congenital transmission of BVDV 2 occurred

in this study, and resulted in fetal deaths, abortions, stillbirths, and PI lambs. Virus was isolated from the live PI lamb WBC on numerous occasions. Virus also was isolated from stillbirths (Scherer et al., 2001).

Table 3: Summary of Characterizations of Experimental Exposure Studies of Sheep to BVDV

Author/year	Clinical signs	Viremia	Nasal Shedding	Hematology	Ab	Route of exposure	Comments
Hewicker/1994	none	not done	N/A	N/A	N/A	IN	fetuses harvested 10-14 days after inoculation were + BVDV brain
Bruschke/1996	none	not done	not done	N/A	N/A	IN	fetuses harvested 2 & 4 wks. after exposure: VI + multiple organs
Bruschke/1996	none	not done	not detected	N/A	N/A	PI	IN & PI exposure compared in post-mortem fetuses: similar distribution of BVDV
Swasdipan/ 2001	none	2/9 viremic on day 4 serum	not done	N/A	N/A	IN	samples of reproductive tissue and fetuses harvested at 4 days: all VI +
Scherer/2001	< 1/3 transient fever;	12/19 (+) WBC during 2-12 day post-inoc sampling period	not done	N/A	15	IN	pregnant ewes exposed: PI produced; stillbirths VI (+)

Ab= antibodies; N/A= not applicable; Y= yes; IM= intramuscular; IN= intranasal; PI= persistently infected; VI= virus isolation

Experimental Exposure of Non-Domestic Ruminants to BVDV

Experimental studies with BVDV and non-domestic ruminants have been performed. Five fawns, 4 mule deer and one white-tail deer, were intranasally inoculated with NY-1 BVDV (Van Campen et al., 1997). None of the inoculated deer showed any signs of disease and daily rectal temparatures remained within normal limits for captive

restrained wild animals during the 3 month period after inoculation. Leukocyte counts were performed in order to assess the degree of immunosuppression, but no consistent changes were noted. Sampling for virus isolation occurred on days 0, 2, 4, 6, 8, 15, 22, 29, and 98. Virus isolation on WBC revealed the presence of virus in 4 out of 5 fawns during the first 15 days after exposure. One fawn was positive in WBC and nasal swabs for days 4, 6 and 8. Virus isolation on nasal swabs were positive in 3 out of 5 animals from day 4 to 8 after infection. The earliest virus isolation was on day 2 after inoculation, and the latest was 15 days after inoculation. The greatest number of animals was positive on day 6. By day 21, all animals in the study had developed a four-fold rise in antibody titer to BVDV. At the conclusion of the 3 month study animals were euthanized and tissues were tested for the presence of BVDV; all samples were negative (Van Campen et al., 1997).

Elk have been experimentally infected with BVDV, and have even transmitted virus to naïve cohorts (Tessaro et al., 1999). Two groups of 5 elk yearlings were experimentally intranasally inoculated with cytopathic BVDV 1 Singer isolate or noncytopathic BVDV 2 24515 isolate in order to determine susceptibility and degree of clinical manifestions of genotypes. Two days after inoculation, a naïve elk was placed in each challenge group. All animals were monitored twice daily for overt signs of clinical disease and blood samples, rectal swabs, and nasal swabs were collected twice weekly for the first 2 weeks, then once a week. Animals were euthanized between days 57-78. All elk remained clinically healthy throughout the study with the exception of the BVDV 2 group contact elk, which died on day 12 after infection, apparently not due to BVDV. Virus was isolated from serum and nasal swabs (all on days 3 and 7 after inoculation)

from all five BVDV 1 inoculated elk. Furthermore, the in-contact elk for the BVDV 1 group was found to have virus in serum on day 23 after inoculation. BVDV was isolated from serum or nasal swabs of only 3 of the 5 BVDV 2 inoculated elk. Virus was not isolated from the in-contact elk, but it died by day 13 post-infection. Virus was not isolated from any of the leukocytes of the inoculated or in-contact elk. All rectal swabs and necropsy tissues were negative for BVDV virus. White blood cell counts for the elk remained within normal limits. In order to test the virulence of the respective isolates, they were inoculated intranasally into two seperate bovine cows. The BVDV 1 inoculated bovine cow showed no clinical signs of disease, no viremia was detected in WBC, serum, or nasal swabs, and WBC counts remained within normal limits. The BVDV 2 inoculated bovine cow developed leucopenia by day 3 after inoculation, which dropped to a low point on day 14 after infection. Differentials showed that initial lymphopenia starting around day 3 and profound neutropenia on days 10 and 14 contributed to the leucogram. Concurrent thrombocytopenia was detected on day 14 after infection. Neutrophil numbers were increasing by day 16, the day of euthanasia. All elk and cattle had produced antibodies to BVDV by day 14 after infection. The BVDV 1 in-contact elk also seroconverted.

Evidence of the ability of BVDV to transplacentally infect the fetus in deer exists (Passler et al., 2007). Nine BVDV seronegative white-tail deer were inoculated intransasally with noncytopathic BVDV 1b BJ strain and BVDV 2 PA131 strain on day 50 of gestation, with some variation. A seronegative buck was placed with the does for breeding purposes. All does were confirmed pregnant by ultrasound on the day of inoculation. No clinical signs were seen in any inoculated does, and no aborted fetuses

were seen. Only one doe carried the pregnancy to term, and had one live fawn and one mummified fetus. The live fawn tested negative for neutralizing antibodies to BVDV, and was positive for BVDV on serum, WBC, immunohistochemistry, and RT-RCR, and was confirmed to be a PI animal. Analysis of the isolate revealed a sequence consistent with BVDV 2 PA131, the inoculum isolate. All does were humanely euthanized at this point, and all were found to have antibodies to BVDV, furthermore, the contact non-inoculated buck was found to be seropositive. There was a higher antibody titer against BVDV 2 detected in all animals, perhaps reflective of advantageous replication strategies or greater host adaptation (Passler et al., 2007).

Transmission of BVDV from PI cattle to naïve deer has been reported (Passler et al., 2009). Seven female seronegative deer were exposed to 2 PI animals of BVDV 1b around 50 days of gestation. After 60 days of cohabitation, the PI animals were withdrawn. Nine live fawns and two stillborn fetuses were born. Samples were collected on all fawns on the day of birth to test for the presence of BVDV. Virus was never detected in the serum of any fawns. Virus was isolated from the WBC of 2 fawns and the nasal swabs of one fawn. Importantly, 3 PI fawns were detected by IHC on skin from ear notch samples. Two stillborn fetuses were also positive for BVDV on IHC. Antibodies to BVDV were detected in all fawns which were not positive for virus isolation. The 7 does and buck also seroconverted to BVDV. No virus was detected in any adult animals.

Table 4: Summary of Characterizations of Experimental Exposure Studies of Non-domesticated Ruminants to BVDV

Author/year/ species	Clinical signs	Viremia	Nasal Shedding	Hematology	Ab	Route of exposure	Comments
VanCampen/ 1997/deer	none	detected day 2 to 15; most animals + on day 6;	4, 6, 8	no changes noted	21	IN	viremia detected in WBC
Tessaro/1999/ elk	none	BVDV 1: 5/5 elk d3, 7 BVDV 2: 3/5 elk d3, 7 (BVDV 1 group in contact elk VI + serum day 23)	3, 7	no changes noted	14	IN	2 challenge groups; naïve contact elk placed in groups on d2; VI unsuccessful on all WBC
Passler/2007/ deer	none	not done; (in contact buck with inoculated does seroconverted)	N/A	N/A	Y	IN	live PI fawn produced
Passler/2009/ deer	none	not done; (In contact buck seroconverted)	N/A	N/A	Y	PI	3 PI fawns produced

Ab= antibodies; N/A= not applicable; Y= yes; IM= intramuscular; IN= intranasal; PI= persistently infected; VI= virus isolation

Evidences of BVDV in New World Camelids: Serological Studies

Serological studies reveal numerous reports of New World Camelids being seropositive to BVDV when cohabitated with ungulates. It has been proposed that close proximity and population dynamics could allow interspecific transmission of a pestivirus (Nettleton, 1990). This concept of interspecific transmission has been echoed and evidenced by serological studies that revealed a number of New World camelids being seropositive when in close proximity to cattle or goats (Belknap et al., 2000; Wentz et al., 2003; Mattson et al., 2006). Around 2% of 390 llamas in Argentina tested positive for virus neutralizing antibodies to BVDV; all of these positive animals were from herds with bovine or ovine co-grazers (Puntel et al., 1999). The seroprevalence rate for llamas

tested in Oregon, U.S.A. was 4.4% of 270 sampled; more than half of these positive animals were from a farm where llamas grazed with cattle (Picton, 1993). An 11% seroprevalence for BVDV antibodies was identified in alpacas in Peru that had been cohabitated with sheep and cattle (Rivera et al., 1987). Wentz reported the presence of antibodies to BVDV in llams, and attributed it to intimate cattle contact (Wentz et al., 2003).

There is mounting evidence that intraspecific spread now may also be occurring in alpacas (Evermann, 2006; Topliff et al., 2009). Suggestively, to date, BVDV 1b remains the most predominant genotype isolated from alpacas (Byers et al., 2009; Carman et al., 2005; Foster et al., 2005; Foster et al., 2007; Goyal et al., 2002) and herds with PI cria exposure have documented seroconversion in adults (Carman et al., 2005; Topliff et al., 2009). In a new study in the U.S.A., sequencing data from 46 BVDV isolates from alpacas placed all isolates as BVDV 1b. Of the 46 isolates, 45 shared greater than 99% nucleotide identity in the 290 base pair 5´ untranslated region. The exception, a Canadian isolate, shared 96.5% homology. This new data points toward maintenance of a unique genotype of BVDV 1b in the alpaca population (Kim et al., 2009). Kim et. al. give two theories for the presence of this phylogenetically unique BVDV 1b in alpacas. One theory is that mere chance resulted in a BVDV 1b PI cria, which propogated the disease through movement and contact with naïve alpaca herds. The second theory suggests that only unique BVDV 1b genotypes can accomplish transplacental infections to result in a PI cria (Kim et al., 2009). However, Edmondson et. al. (personal communication) experimentally inoculated pregnant alpacas simeultaneoulsy with BVDV 1b cattle, BVDV 1b isolated from an alpaca, and BVDV 2.

Sequencing data from PI crias revealed the presence of BVDV 1b cattle and BVDV 1b alpaca strain, no BVDV 2 was recovered. A recent prevalence study of 63 alpaca herds in the USA found a 6.3% herd-level incidence of PI's and a 25.4% seropositivity to BVDV. With herd-level prevalence PI rates in beef cattle herds ranging from 3% (Wittum et al., 2001) to 4% (O'Connor et al., 2007), an alpaca herd-level incidence PI rate of 6.3% seems high. Contributing factors for the increased herd prevalence of PI alpacas could be related to the existing low prevalence of seropositive alpacas (indicating low amounts of previous exposure to BVDV) and increased susceptibility and/or frequent movement of alpacas (dams with crias by their side) for breeding (Topliff et al., 2009). Of the four BVDV isolates obtained from PI herds in Topliff's study, three were found to have close homology (96.4 to 97.2%) with NY-1 (a BVDV 1b) and 1 was found to have close homology (97.6%) with NADL (a BVDV 1a) (Topliff et al., 2009). This study remains to be the only study that indicates infection of alpacas with BVDV 1a, while other literature continues to confirm the presence of only BVDV 1b in alpacas. This study by Topliff and others concluded that both the presence of PIs and the use of bovine and caprine colostrum contributed to the number of seropositive animals in a herd (Topliff et al., 2009).

Evidences of BVDV in New World Camelids: Case Reports

The number of case reports of BVDV and alpacas are increasing and evidence demonstrates that alpacas can contract and propagate the pathogen. Belknap et. al.

detected BVDV in a stillborn cria from an obese llama. At a separate farm, postmortem testing of a pregnant llama and a 15 month-old male llama revealed the presence of BVDV; these animals had no contact with cattle, but exposure to Angora goats and exotic sheep. In the three aforementioned cases, immunoperoxidase staining of the lungs, liver, spleen and thymus (15 month-old) yielded the positive results (Belknap et al., 2000). The first successful isolation of the BVDV from alpacas involved propagation of a noncytopathic BVDV 1b from a stillborn alpaca in Minnesota in 2002 (Goyal et al., 2002). In 2005, a 7-month-old, ill-thrift alpaca in the U.K. was found to be positive for BVDV 1b (Foster et al., 2005). A survey of four flocks (80 animals) suspected to be infected with BVDV around metropolitan Chile resulted in isolation of virus from 18 South American Camelids. Molecular characterization found BVDV 1 in six alpacas, and BVDV 2 in four alpacas and eight llamas. These positive samples were from eight healthy alpacas, two alpacas with history of abortion, five healthy llamas, two llamas with history of abortion, and one dead llama (Celedon et al., 2006).

The first clinical outbreak of the disease occurred in 2005 when 9 adult alpacas exhibited vague signs of lethargy, anorexia, and depression 2.5 months after the addition of a chronically ill cria to the farm (Carman et al., 2005). Seventeen of twenty adult alpacas subsequently seroconverted and one alpaca aborted a fetus from which BVDV 1b was isolated. A PI cria was born nine months after clinical illness in the adults; BVDV 1b was isolated from this cria. The seroconverted adults had antibodies to BVDV 1a (NADL) and BVDV 2 (NVSL 125c), possessing higher titers to BVDV 1, the greatest of which was 1:3072 (Carman et al., 2005). In 2006, the second case of clinical disease in alpacas appeared and involved a premature alpaca cria that exhibited signs of intermittent

pyrexia, chronic upper respiratory infections, and failure to thrive. Bovine viral diarrhea virus 1 was isolated from this cria and a diagnosis of persistent infection was made. This cria exposed other alpacas to BVDV during its life, and BVDV was isolated from white blood cells of one acutely infected male. Furthermore, a pregnant alpaca exposed at 49 days of gestation later gave birth to a PI cria. Fourteen out of fifteen exposed adult alpacas possessed antibodies against BVDV 1 (log-transformed mean serum VN antibody titer of 1:280) and BVDV 2 (log-transformed mean serum VN antibody titer of 1:94) (Mattson et al., 2006). In the United Kingdom, three crias with a history of illthrift and diarrhea were found to be positive on multiple tests for BVDV 1b on numerous tissues collected postmortem, and BVDV antibodies were found in 16 out of 25 herd members. The source of the infection was unclear (Foster et al., 2007). Recently, a 4-month-old cria with a history of anorexia, cachexia, and failure to thrive was reported positive for BVDV 1b in Washington, USA. This animal was positive for virus on multiple tissues, including various organs, and, most notably, salivary glands and testicles. The presence of virus in these secretions may play a role in virus transmission as alpacas will spit for both social and reproductive receptivity communication (Byers et al., 2009).

It was previously speculated that viremia with BVDV seldom developed in camelids (Wentz et al., 2003). However, due to the increasing number of confirmed PIs in New World camelids, it is apparent that viremia is indeed occurring with certain strains of BVDV (Mattson et al., 2006).

There is a paucity of knowledge of infectivity, virulence, or host interactions of the different strains of BVDV in alpacas; whereas, characterization of virologic, serologic, and clinical results of experimental infection are well documented in cattle

(Baker, 1995; Thurmond, 2005). In cattle, acute infections are generally subclinical but may cause anorexia, lethargy, respiratory disease, decreased milk production, mucosal lesions, and even decreased fertility and early embryonic death (Baker, 1995; Fray et al., 2000; Grooms, 2004; Kafi et al., 2002; McGowan et al., 1993; McGowan & Kirkland, 1995; Virakul et al., 1988). As discussed previously, the majority of post-natal cattle exposed to BVDV have been shown to undergo a period of transient immunosupression based on complete blood counts, even in the absence of clinical signs (Potgieter, 1995).

The acutely infected animal may be epidemiologically important within the alpaca industry as travel and cohabitation are required for breeding management, shows, and social gatherings. Of course, any existing PI crias remaining with their dam during these times would also disseminate BVDV. In cattle, the acutely infected animal remains important not only due to the immunosuppressive and direct effects of the virus on the host animal but also the possible ability of this animal to shed virus to other animals (Thurmond, 2005).

A study investigating the potential importance of the transiently or acutely infected animal in epidemiological models for BVDV in cattle was conducted in 1992 (Houe, 1992). This study examined the incidence of PI animals in twelve separate herds with clinical BVDV and compared this to ten herds with no evidence of BVDV and examined the incidences of PI animals born after the oldest PI animal. The PI animals were found to occur in different age groups, and temporal statistical analysis led to the conclusion that it remained likely that separate episodes of acute infection were occurring in select cow herds that were enrolled in the study. Based upon the birth of the PIs, the acute infections appeared to be of short duration and only infected a particular subset of

herd members before transmission of virus ceased. Testing revealed that the infections must have been introduced by means other than a PI animal and placed importance on the acutely infected animal (Houe, 1992).

In cattle, there is evidence that acutely infected animals may be able to maintain the virus within a population (Smith & Grotelueschen, 2004). Another BVDV study was executed in order to examine the herd incidence of BVDV infections in herds previously without PI animals (Houe & Palfi, 1993). Incidence risk calculations seemed to suggest that the introduction of new animals seemed to be correlative with reinfection rates. With relatively few new introductions in this particular herd study, the authors speculated it was highly unlikely that all new disease was solely sequelae to the purchase of PI animals, and that reinfections might be caused by introduction of acutely infected animals (Houe & Palfi, 1993).

Other epidemiological studies yield evidence of the ability of the acutely infected animal to transmit BVDV. In one study, BVDV testing was conducted for three years in a large, controlled, well-managed dairy herd (Moerman et al., 1993). The spread of virus was monitored in areas of the farm determined to possess a PI animal, and in areas of the farm said to possess only transiently infected animals. The transmission of virus was documented based upon the appearance of antibodies in naïve animals moved to the areas of the farm that only possessed transiently infected animals. Evidence of transmission by transient infection was supported by the slow rate of seroconversion seen in these naïve animals. In contrast, naïve animals placed in contact with the PI animals exhibited a more rapid seroconversion. Furthermore, the numbers of naïve animals that developed

antibodies to BVDV was greater in the naïve animals exposed to the PI animal (Moerman et al., 1993).

Authors have reported controlled clinical studies in which transmission was not effective in acutely infected animals, but still recommend implementing control plans to address acutely infected animals. A group of five calves placed in nose-to-nose contact with a clinically healthy PI calf contracted BVDV (Niskanen et al., 2000). The PI was determined to have noncytopathic BVDV 1. Fourteen calves were then introduced to the transiently infected calves in groups of two on days 4, 7, 14, 21, 28, 35, and 42. No virus was detected in these 14 calves, nor did they display any clinical signs. No antibodies to BVDV were detected in calves during the 28 days after exposure. Although seemingly ineffective transmitters based on this experiment, transiently infected animals should not be eliminated from a control plan for BVDV due to strain variance in virulence and underlying host factors (Niskanen et al., 2000). For example, cytopathic BVDV 1d has been effectively transmitted from experimentally acutely infected animals to naïve cohorts (Baule et al., 2001). In this study, the contact calves were placed in with experimentally infected calves on the day of infection and developed clinical symptoms on days 11 to 12. Virus was isolated from tissues harvested from these exposed control calves (Baule et al., 2001). With this evidence, it seems that the acutely infected animal could very well play a role in the epidemiology of BVDV in alpacas as well. Experimental studies to elaborate on transmission of BVDV in alpacas are limited, but follow.

Experimental Exposures of New World Camelids to BVDV

The single published study involving experimental exposure of New World Camelids to BVDV is from 2003, in which the purpose of this study was to determine the effect of experimental BVDV inoculation, categorize the genetic classification of BVDV isolates from llamas, and perform a seroprevalence study for BVDV in llamas. Four pregnant seronegative llamas, two of 102 days of gestation and two of 68 days of gestation were experimentally inoculated with a BVDV of unknown genotype that was isolated from a llama.

Cell cultures used were bovine turbinate cells (BTU) and Madin Darby bovine kidney cells (MDBK). Virus detection was performed by use of an immunoperoxidase staining technique. A polyclonal antibody test against NADL (a BVDV 1a), NY-1 (a BVDV 1b), and SD-1 (a BVDV 1a) was used for the virus neutralization testing. Experimental inoculation 5 ml of 7.5 X 10^5 $TCID_{50}$ of BVDV isolated from a llama was performed by intranasal nebulization. After inoculation, daily physical exams and collection of serum, whole blood, and nasal swab samples on days 0, 3, 5, 7, 8, 9, 10, and 14 after inoculation was performed. For the isolation of white blood cell (WBC) fraction, EDTA tubes were spun 150 X g at 4 °C for 1 hour, followed by isolation of the WBC fraction. The fraction was then suspended in 0.15 M ammonium chloride, pelleted, and resuspended in MEM. After centrifugation was repeated, the buffy coat cells were resuspended in 0.5ml of MEM. Nasal swabs were collected by placement of a polyethylene fiber tipped swabs into each nostril and then placing the swab into 1ml of

MEM which contained 1% fetal bovine serum, gentamicin, amphotericin B, and sodium penicillin G.

Wentz's post-natal inoculation of BVDV into New World Camelids did not induce any clinical signs. Wentz's study did not include white blood cell counts, and it remained unknown whether New World camelids undergo a period of immunosuppresion after exposure to BVDV as demonstrated in experimental inoculations in cattle (Potgieter, 1995; Baker & Houe, 1995; Kapil et al., 2005). A transient viremia was detected, in which two animals were positive for virus in serum from day 3 to 10 after inoculation, and two animals were positive for day 10 after inoculation. Isolation of virus from white blood cells was detected in three out of four animals: in two llamas on days 3 and 5 after inoculation and in one llama on day 7 after inoculation. Due to cytotoxicity, no nasal swab samples were tested for virus isolation. One animal possessed antibody titers of 1:10 on day 14 after inoculation, and the remainder of llamas had antibody titers on day 30 after inoculation ranged from 1:20 to 1:160.

Based upon the low numbers of buffy coat samples found to be virus isolation positive, Wentz concluded and echoed the comments of Mattson (Mattson, 1994) in that "BVDV replicates to a limited extent in the white blood cells of New World camelids (Wentz et al., 2003)." With these statements in mind, experimental studies with BVDV and New World camelids remained absent until case reports of BVDV positive alpacas began to appear in 2005.

Sequencing data from this study revealed that the llama inoculum was closest to Singer isolate, a BVDV 1a. Analysis of three other llama isolates revealed the following: one shared homology with Singer isolate (a BVDV 1a), one shared homology with

NADL isolate (a BVDV 1b), and one shared homology with 9762 isolate (a BVDV 1b). Detection of similarity in the 5´ non-translated region of llama and bovine isolates led to the conclusion that cattle were the most likely source of BVDV in New World camelids.

III. COMPARISON OF CLINICAL, HEMATOLOGICAL, AND VIROLOGICAL FINDINGS IN ALPACAS (LAMA PACOS) INOCULATED WITH *BOVINE VIRAL DIARRHEA VIRUS* ISOLATES OF ALPACA OR BOVINE ORIGIN

Abstract

Clinical evidence demonstrates that alpacas may contract and propagate bovine viral diarrhea virus (BVDV). The objective of this research was to compare and characterize clinical signs, hematological findings, viremia, and seroconversion resulting from intranasal inoculation of alpacas with BVDV 1b and BVDV 2 isolates from cattle and a BVDV 1b isolate of alpaca origin. Three groups of six alpacas were intranasally inoculated with a different isolate (Group 1: BVDV 1b of bovine origin; Group 2: BVDV 2 of bovine origin; Group 3: BVDV 1b of alpaca origin). Following inoculation, all three genotypes induced viremia, nasal shedding and seroconversion in naïve alpacas. The onset of viral detection in serum was significantly different among groups; the mean onset was 4.0, 2.3, and 7.5 d for Groups 1, 2, and 3, respectively. Onset and duration of viral detection in white blood cells was significantly different with onset at 3.0, 2.3, and 4.7 d, and cessation at 13.0, 10.0, and 12.3 d, respectively. The mean onset of viral nasal

shedding was 6.9 d and was not significantly different between groups. Virus was detected after inoculation until an average of 8.9 d in nasal secretions and 9.4 d in serum. A reduction in mean total leukocytes was observed in all three groups when compared to pre-inoculation leukograms. Results demonstrate that BVDV 1b and 2 strains cause alpacas to exhibit viremia and nasal shedding of virus in a temporal pattern that is similar to the outcome of acute infection of cattle.

Keywords: Bovine Viral Diarrhea Virus; cattle; alpacas

Introduction

Bovine viral diarrhea virus (BVDV) is one of the most important infectious agents in the cattle industry and causes substantial economic losses worldwide (Grooms, 2004). Belonging to the family Flaviviridae, the *Pestivirus* genus consists of the following positive-sense RNA viruses: BVDV 1, BVDV 2, classical swine fever virus, and border disease virus (Ridpath, 2005). The virus is divided based on genetic sequencing into BVDV 1 and BVDV 2 and further divided into subgenotypes (Ridpath, 2005). The virus is segregated into biotypes based upon its cytopathic effects during cell culture. Either genotype (1 or 2) may exist as a cytopthic or noncytopathic strain, with the latter biotype dominating in nature (Bolin & Grooms, 2004).

The clinical signs that BVDV induces in its host are diverse yet well documented in cattle. Basically, infections are differentiated as acute or persistent (Baker, 1995). The acutely infected animal is characterized by postnatal exposure to the virus and subsequent clearance of the virus by the host's immune system (Evermann & Barrington, 2005).

This acutely infected animal may serve as a temporary source of viral transmission, but dissemination of virus predominantly occurs through persistently infected (PI) animals. A PI calf develops when a fetus is exposed to a noncytopathic biotype of the virus prior to approximately 5 months of gestation. The developing fetus recognizes the viral genome as self and subsequently sheds high numbers of virus particles throughout life (Brock et al., 2005). Virus may be found in saliva, tears, nasal secretions, vaginal secretions, feces, urine, milk, and semen (Thurmond, 2005). The PI animal prevalence is between 0.5% to 2% of the cattle population (Houe, 1995). Most resources for the cattle industry are primarily focused on identifying and eliminating PI animals in order to stop the spread of disease (Grooms, 2004).

Originally, pestiviruses were categorized by the species of animal infected; however, in recent years it has become clear that BVDV may infect species other than cattle (Ames, 2005). Bovine viral diarrhea virus has been isolated in natural settings from pigs (Terpstra & Wensvoort, 1988; Paton & Done, 1994), sheep (Carlsson, 1991; Paton & Done, 1994), kids and lambs (Nettleton, 1990), deer (Frolich, 1995; Frolich & Hofmann, 1995; Nettleton et al., 1980; Van Campen et al., 2001), old-world camels (Hegazy et al., 1996), llamas (Belknap et al., 2000; Motha & Tham, 1992), and alpacas (Byers et al., 2009; Carman et al., 2005; Celedon et al., 2006; Foster et al., 2005; Foster et al., 2007; Goyal et al., 2002; Mattson et al., 2006; Topliff et al., 2009).

Recent isolations of BVDV from alpacas have been described, and evidence demonstrates that alpacas may contract and propagate the virus. Belknap et. al. identified BVDV in a stillborn cria from an obese llama (Belknap et al., 2000). The first successful isolation of BVDV from alpacas involved propagation of BVDV 1b from a stillborn

alpaca in 2002 (Goyal et al., 2002). In 2005, a 7 month old ill-thrift alpaca was found to be positive for BVDV 1b (Foster et al., 2005). A survey of 4 flocks (80 animals) suspected to be infected with BVDV around metropolitan Chile resulted in isolation of virus from 18 South American camelids, and molecular characterization identified BVDV 1 in 6 alpacas, and BVDV 2 in 4 alpacas and 8 llamas (Celedon et al., 2006). These BVDV-positive samples were from 8 healthy alpacas, 2 alpacas with history of abortion, 5 healthy llamas, 2 llamas with history of abortion, and 1 dead llama.

The first clinical outbreak of BVDV-associated disease in alpacas was reported in 2005 when 9 adult alpacas exhibited vague signs of lethargy, anorexia, and depression 2.5 months after the addition of a chronically ill cria to the farm (Carman et al., 2005). Seventeen of the adult alpacas subsequently seroconverted and one alpaca aborted a fetus from which BVDV 1b was isolated. A PI cria was born 9 months after clinical illness was seen in the adults (Carman et al., 2005). The second case of clinical disease in alpacas involved a premature alpaca cria that exhibited signs of intermittent pyrexia, chronic upper respiratory infections, and failure to thrive (Mattson et al., 2006). Bovine viral diarrhea virus 1 was isolated from this cria and a diagnosis of persistent infection was made. This cria exposed other alpacas to BVDV during its life and a pregnant alpaca exposed at 49 days of gestation later gave birth to a PI cria (Mattson et al., 2006). Both of the aforementioned case reports included serological profiles conducted on exposed alpacas and both revealed antibodies to BVDV 1 and 2 (Carman et al., 2005; Mattson et al., 2006). In the United Kingdom, three crias with a history of ill-thrift and diarrhea were found to be positive for BVDV 1b on numerous tissues collected postmortem, and antibodies to BVDV were found in numerous herd members (Foster et al., 2007).

Recently, a 4-month-old cria with a history of anorexia, cachexia, and failure to thrive was reported positive for BVDV 1b in Washington, USA (Byers et al., 2009).

Despite previous speculation that viremia with BVDV seldom developed in camelids (Wentz et al., 2003), the increasing number of confirmed PIs in New World camelids demonstrates that viremia may occur with certain strains of BVDV (Mattson et al., 2006). To date, there is a paucity of knowledge regarding infectivity, virulence, and host interactions of different strains of BVDV in alpacas; whereas, characterization of virologic, serologic, and clinical results of experimental infection are well documented in cattle (Baker, 1995; Thurmond, 2005). At present, only a single study involving experimental inoculation of New World camelids has been performed, and in this study, 4 adult, pregnant llamas received a noncytopathic isolate of BVDV of unknown genotype that had been isolated from a llama. Because of this lack of data on BVDV infections in alpacas, the objective of this research was to compare and characterize clinical signs, viremia, nasal shedding, and seroconversion resulting from intranasal inoculation of alpacas with BVDV 1b and 2 isolates of bovine origin and a BVDV 1b isolate of alpaca origin.

Materials and Methods

Animals

Seventeen male, castrated alpacas and one female alpaca were used in this study. All animals were virus isolation negative and seronegative to BVDV and were housed at Auburn University College of Veterinary Medicine until the study began. Alpacas were randomly separated into groups containing six alpacas each, and only one group was

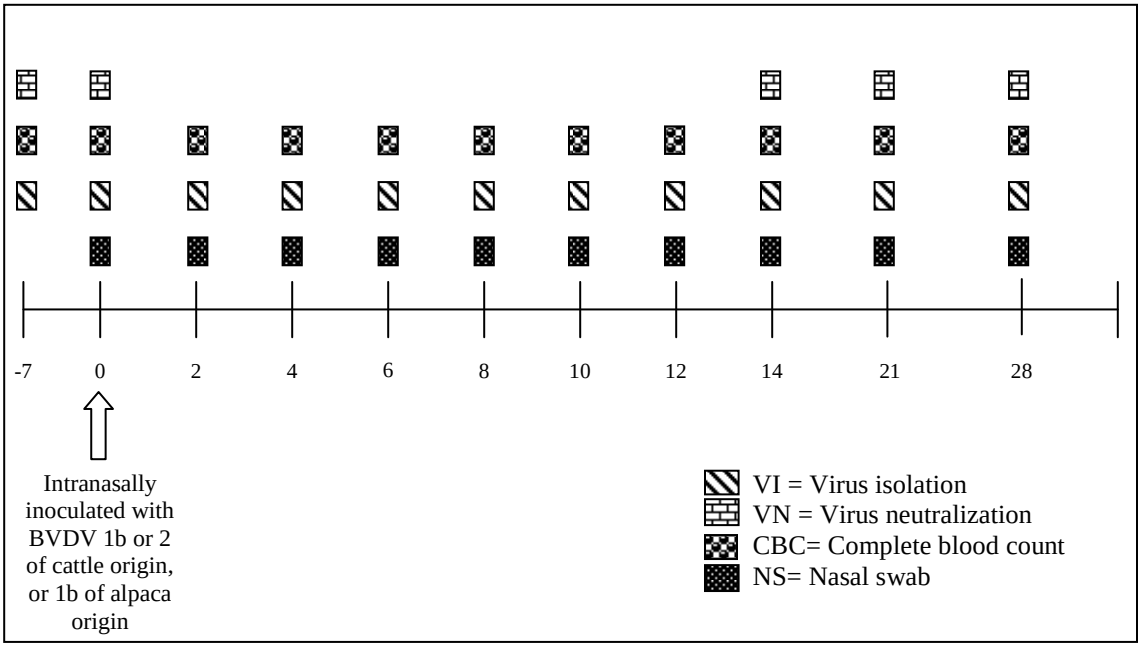
inoculated at a time in order to prevent possible cross-contamination between groups. Seven days prior to exposure, alpacas (n=6) were moved to isolated pastures at the North Auburn BVDV Unit. All animals were supplied water and hay *ad libitum.* Grain supplementation was supplied as needed based upon body condition scoring. The alpacas were sheared for the summer months and had unlimited access to shade. Animals were monitored daily for detection of any clinical signs of disease. Heart rate, temperature, and respiratory rates were performed on alternate days during the study period. All procedures described were performed with the approval of the Auburn University Institutional Animal Care and Use Committee protocol number 2007-1237.

Experimental Design

The objectives of this project were to characterize the virologic, clinical, and serologic findings induced by experimental exposure of naïve alpacas to various genotypes of BVDV. Seven days prior to inoculation, virus isolation, virus neutralization, and complete blood counts were performed on animals entering the study (Fig. 1). On study day 0, three groups of six alpacas each were intranasally inoculated with BVDV (Group 1: BVDV 1b of bovine origin; Group 2: BVDV 2 of bovine origin; Group 3: BVDV 1b of alpaca origin).Alpacas were inoculated by intranasal nebulization using a DeVilbiss atomizer model 163 (Sunrise Medical Inc., Fort Pierce, CA, USA) with 5 mL of cell culture supernatant that contained 6.2×10^6 $CCID_{50}$/mL of the noncytopathic, BVDV 1b strain BJ (cattle origin), 1.9×10^7 $CCID_{50}$/mL of the noncytopathic, BVDV 2 strain PA 131 (cattle origin), or 6.2×10^4 $CCID_{50}$/mL of the noncytopathic, BVDV 1b strain isolated from a PI alpaca. Virus isolation was performed

on study days -7, 0, 2, 4, 6, 8, 10, 12, 14, 21, 28 from serum, white blood cell, and nasal swab samples. Daily physical examinations were performed and serial leukocyte counts were evaluated on the above sample days. Virus neutralization assays were performed to detect seroconversion on study days -7, 0, 14, 21, 28.

Figure 1: Experimental Design: exposure of naïve alpacas to bovine viral diarrhea virus isolated from cattle and alpacas



Virus

Original plans included exposure of alpacas to a BVDV 1a. Thus, the BJ strain was selected and obtained for this challenge based on previous published literature (Brock et al., 2006). However, upon conclusion of the study, sequencing of the 5' nontranslated region of the viral genome revealed that the BJ strain used in this study was a BVDV 1b. Thus, the challenge viruses, BVDV 1b (BJ) and BVDV 2 (PA-131) of

cattle origin were grown in Madin Darby bovine kidney (MDBK) cells cultured in minimum essential medium (MEM) with Earle's salts supplemented with 10% (v/v) equine serum, sodium bicarbonate (0.75 mg/mL), L-glutamine (0.29 mg/mL), penicillin G (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL; complete MEM). The BVDV 1b isolated from alpacas (108417.206; generously provided by Dr. Ed Dubovi) was grown in bovine turbinate cells (BTU) with complete MEM.

Virus Isolation

Virus isolation was performed on buffy coat, serum, and nasal swab samples as described previously (Walz et al., 2008; Givens et al., 1999). Serum samples were assayed for BVDV by passage in MDBK cells, with the exception of samples from animals infected with the BVDV 1b alpaca strain, in which BTU cells were used. A 6-well plate that had been seeded 24 h earlier with MDBK cells in complete MEM was inoculated with 768 µL of serum sample diluted in 192 µL of MEM or 960 µL of buffy coat or nasal swab sample. Following a 1-h adsorption period, 3 mL of complete MEM was added. The plates were incubated for 5 days at 38.5°C in a humidified atmosphere of 5% CO_2 and air. Following a single freeze-thaw cycle to release intracellular virus, lysates from this procedure were assayed in triplicate by diluting 10 µL of cell lysate with 90 µL of culture medium and subsequently adding 50 µL of culture medium containing MDBK (for cattle origin strains) or BTU cells (alpaca 1b strain) to the wells of a 96-well culture plate. Following incubation for 72 h at 37°C in humidified air containing 5% CO_2, the MDBK or BTU cells were stained for BVDV antigen by an immunoperoxidase

monolayer assay using the BVDV-specific monoclonal antibodies D89 and 20.10.6 (Givens et al., 2003).

Sample Collection and Processing

Blood was collected in sterile sodium ethylenediamine tetraacetic acid (EDTA; purple top) blood tubes and virus isolation was performed on buffy coat samples. White blood cell isolation was performed as described previously (Walz et al., 2008) with a few modifications. Briefly, whole blood collected in EDTA was processed to yield the buffy coat cells. Following centrifugation of the whole blood at 200 x g for 30 min, the buffy coat cells were removed. Lysis of contaminating red blood cells was performed using 0.15 M ammonium chloride (NH4Cl) at 4°C for one hour. The buffy coat cells were washed in 10 mL of complete MEM. After centrifugation at 300 x g for 10 min, the buffy coat cells were resuspended in 1.0 mL of MEM to be used in virus isolation procedures. Blood was also obtained in red top tubes (no additive) and virus isolation and virus neutralization was performed on serum. Routine complete blood counts (CBC) were performed in the Auburn University College of Veterinary Medicine Clinical Pathology Laboratory using the Advia 120 Hematology Instrument (Siemens Healthcare Diagnostics, Deerfield, IL; software version 3.1.8.0-MS). Complete blood count measurements included red cell count, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte % and absolute reticulocyte count, total white blood cell, platelet count, and mean platelet volume. A packed cell volume was determined by microhematocrit centrifugation (owing to alpaca erythrocyte elliptical shape, hematocrit reported by Advia

120 is inaccurate). Leukocyte differential (which included segmented neutrophils, band neutrophils, lymphocytes, monocytes, eosinophils, and basophils) and cellular morphologic observations were performed manually from blood smears. Smears were prepared from EDTA-anticoagulated blood on each animal within 30 to 60 minutes of arrival in the laboratory. Smears were stained with modified Wright's stain in an automated stainer (Aerospray 7150 Hematology Slide Stainer-cytocentrifuge, Wescor, Inc., Logan, UT). For quality control, 3-level controls (ADVIA 120 3-in-1 TESTpoint, Siemens Medical solutions Diagnostics, Tarrytown, NY, USA) were performed daily.

Nasal swabs were obtained with a sterile dacron-tipped swab placed into the external nares and rotated five times. The swab was then placed into 3 mL of complete MEM supplemented with 1 mg/mL of gentamicin sulfate. Virus isolation was performed as described above.

Virus Neutralization

The virus neutralization microtiter assay was used to detect antibodies against BVDV in the serum of alpacas (Givens et al., 2002). Sera were obtained from alpacas and were tested for neutralizing antibodies to the three strains of BVDV used in this research. The virus neutralization test was set up in 96-well microtiter plates. After heat inactivation at 56°C for 30 minutes, serial two-fold dilutions, ranging from 1:4 to 1:40,960, were made in triplicate for each serum sample. Each well of a 96-well plate was inoculated with an equal volume (50 μL) of culture medium containing 100 to 300 tissue culture infective dose ($TCID_{50}$) of either the noncytopathic BVDV-1b BJ, noncytopathic BVDV-2 PA131, or noncytopathic BVDV-1b isolated from a PI alpaca.

After inoculation, plates were incubated (38.5°C) in a humidified atmosphere of 5% CO_2 and air for 1 hour. Then, 2.5 x 10^3 MDBK cells (BJ and PA131) or BTU cells (alpaca 1b) in 50 μL of culture medium were added to each well. Plates were incubated for 72 hours and then stained using immunoperoxidase labeled monoclonal antibodies. A simplified endpoint was determined based on the greatest dilution at which 2 of 3 wells were free of virus.

Statistical Analysis

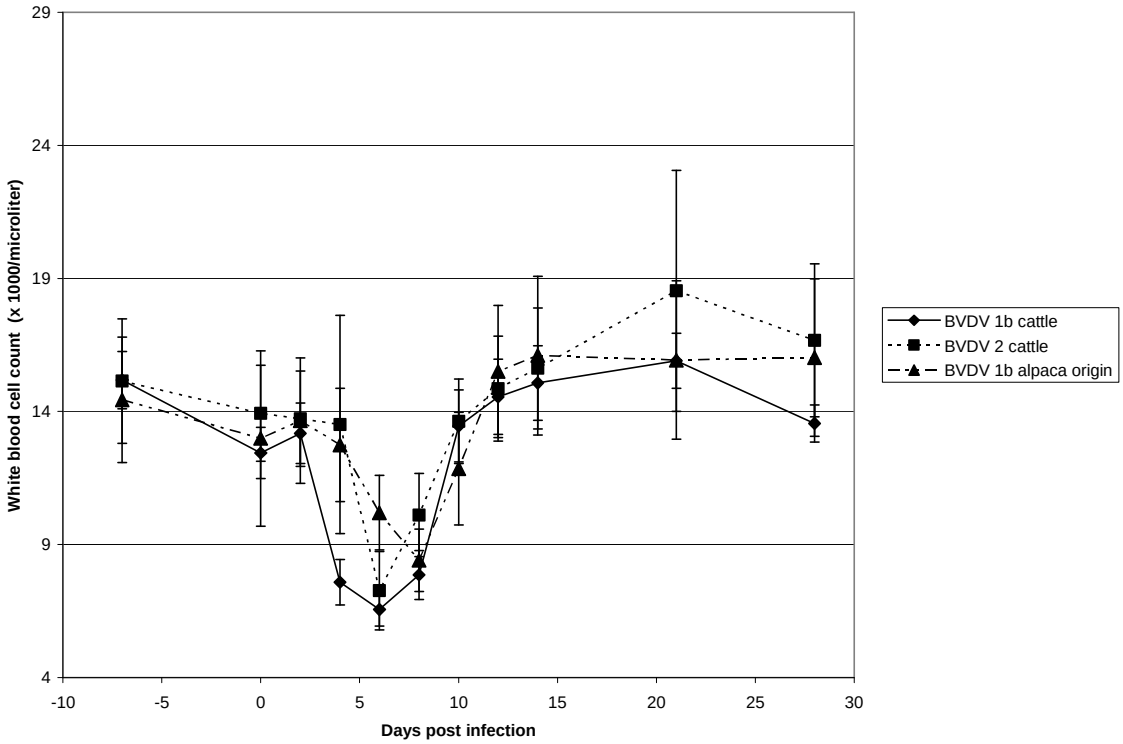
Data for continuous variables measured multiple times over the course of the study were statistically evaluated by use of repeated measures ANOVA. The mixed procedure of the software program (SAS 9.1, SAS Institute Inc., Cary, N.C., U.S.A.) was used to evaluate the effects of group, time, and the interaction between group and time. If the group by time interaction was significant ($P < 0.05$), group effects within time were evaluated between the 3 groups by the least significant difference test. A Dunnett's adjustment for multiple comparisons was used to identify days with significant changes from the day -7 value within groups. For categorical data measurements, results were evaluated using a Chi-squared test. Results were analyzed using JMP software (SAS Institute Inc., Cary, N.C., U.S.A.).

Results

Based upon daily temperature, pulse and respiration parameters, signs of clinical illness were not detected in BVDV-inoculated alpacas. Complete blood counts revealed a reduction in mean leukocyte counts in all three study groups (Figure 2); however,

statistically significant reductions in mean total leukocyte counts were detected only in group 1 between pre-inoculation day -7 and post-inoculation days 4 ($p=0.0003$), 6 ($p<0.0001$), and 8 ($p=0.0006$). The statistically significant differences seen in Group 1 leukocyte counts are correlative with significant drops in mean neutrophil counts between days -7 and days 4 ($p=0.0028$), 6 ($p=0.0016$), and 8 ($p=0.0034$) and significant decreases in mean lymphocyte numbers between days -7 and day 6 ($p=0.0355$). The mean minimum leukocyte count for alpacas inoculated with BVDV 1b BJ (Group 1) was 6,280/μL; for BVDV 2 PA131 (Group 2) was 6,690/μL; and for BVDV 1b alpaca (Group 3) was 8,080/μL. The mean total numerical drop to the minimum leukocyte count for groups was 8,900, 8,450, and 6,360 cells/μL, respectively. A 59%, 56%, and 44% decrease in mean total leukocytes between days -7 mean values and mean minimum leukocyte values was seen in groups 1, 2, and 3, respectively. The respective means for time to nadir between groups was 5.7, 5.7 and 8.0 days, respectively. The nadir of leukopenia occurred significantly later in Group 3 alpacas (BVDV 1b alpaca) as compared to groups of alpacas inoculated with strains of bovine origin ($p=0.021$).

Figure 2: Mean white blood cell counts after experimental intranasal inoculation of naïve alpacas with various genotypes of bovine viral diarrhea virus isolated from cattle and alpacas



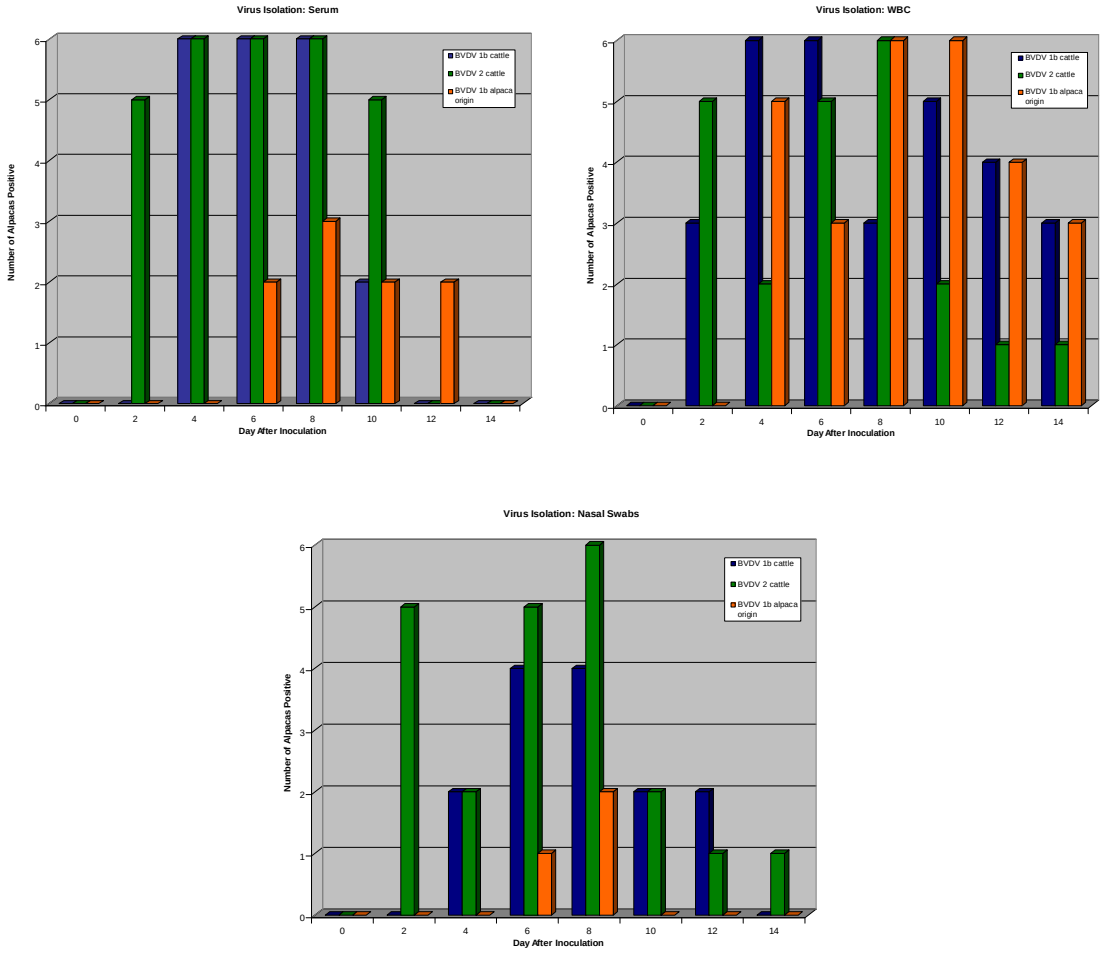
All three genotypes of BVDV induced viremia in naïve alpacas, yet virus isolation results varied between groups (Table 5). The number of alpacas positive for virus isolation in serum, white blood cells, and nasal swabs on respective sample days is depicted in Figure 3. Virus was not isolated from any samples collected on days 21 or 28 after inoculation.

Table 5: Total number of alpacas from which virus was isolated from serum, white blood cells, or nasal swabs after experimental inoculation of naïve alpacas with various

	Serum (positive/total)	White Blood Cells (positive/total)	Nasal Swabs (positive/total)
BVDV 1b cattle	6/6	6/6	6/6
BVDV 2 cattle	6/6	6/6	4/6
BVDV 1b alpaca	4/6	6/6	3/6

genotypes of bovine viral diarrhea virus of cattle or alpaca origin.

Figure 3: The number of alpacas from which virus was isolated from serum, white blood cells (WBC), or nasal swabs after experimental inoculation with various genotypes of bovine viral diarrhea virus (BVDV) of cattle or alpaca origin. BVDV was not isolated from any samples on days 21 or 28 after inoculation.



The onset of viral detection in serum was significantly different among groups ($p=0.0001$); the mean onset was 4.0, 2.3 and 7.5 d for Groups 1, 2, and 3, respectively. Furthermore, the onset of viral detection in white blood cells was significantly different ($p=0.014$) among groups with means of 3.0, 2.3 and 4.7 d, respectively. The mean onset of viral nasal shedding was 6.9 d and was not significantly different ($p=0.444$) among groups. Cessation of viral detection in serum (mean = 9.4 d) and nasal secretions (mean = 8.9 d) was not statistically different among groups ($p=0.296$ and $p=0.354$), but duration of viral detection in white blood cells was significantly different ($p=0.0434$) with detection until means of 13.0, 10.0 and 12.3 d, respectively.

By day 14 post-infection, two animals in Group 1 and two animals in Group 3 had seroconverted. By day 21, all animals in Group 1, two animals in Group 2, and all the animals in Group 3 had seroconverted. By day 28 after infection, all animals in the study had seroconverted with the exception of one animal in Group 2. Table 2 lists the reciprocal of the geometric means antibody titers and the range of the reciprocal of the antibody titers 28 days after inoculation to the specific viral strain with which each group of naïve alpacas was intranasally inoculated. The day 28 antibody titers for Group 2 were significantly lower than detected in Group 3 (Table 6).

Table 6: Reciprocal of the Day 28 geometric means antibody titers and ranges of the reciprocal of the antibody titers to the specific viral strain with which each group of naïve alpacas was intranasally inoculated.

Viral Strain of Inoculation	Geometric Mean	Range
BVDV 1b cattle	91[a,b]	8-1024
BVDV 2 cattle	23[a]	4-128
BVDV 1b alpaca	456[b]	256-1024

Values within the same column lacking the same superscript (a and b) differ ($p<0.05$)

Discussion

All evaluated strains of BVDV induced viremia in alpacas. Our work demonstrates that BVDV 1b and 2 isolated from cattle are as capable as the BVDV 1b alpaca strain at inducing viremia, decreased leukocyte counts, and nasal shedding of virus in alpacas. Characterization of acute BVDV infections in alpacas appears to follow cattle models. In cattle, acute infections are generally subclinical but may cause anorexia, lethargy, respiratory disease, decreased milk production, mucosal lesions, and even decreased fertility and early embryonic death (Baker, 1995; Fray et al., 2000; Grooms, 2004; Kafi et al., 2002; McGowan et al., 1993; McGowan & Kirkland, 1995; Virakul et al., 1988). Cattle that are acutely infected with BVDV appear to shed low numbers of virus particles for 1 to 21 days with most shedding within a time period of 10 days or less (Thurmond, 2005). The acutely infected animal is characterized by postnatal exposure to the virus and subsequent clearance of the virus from the body by the host's immune system (Evermann & Barrington, 2005). Our results concur with prior results from experimental inoculation of llamas with BVDV in which signs of clinical disease were

not observed, but a transient viremia was detected 3 to 8 days after inoculation(Wentz et al., 2003). In that study, duration of nasal shedding of virus for acutely infected llamas was not determined; one llama seroconverted at day 14, while the remaining three llamas seroconverted by day 30 (Wentz et al., 2003). Thus, alpacas are similar to pigs (Walz et al., 1999), sheep (Scherer et al., 2001), elk (Tessaro et al., 1999), deer (Van Campen et al., 1997; Passler et al., 2007) and llamas (Wentz et al., 2003) in their potential for infection with BVDV under controlled experimental conditions.

In our experiment, the numerical differences observed in day 28 geometric mean antibody titers between study groups could be attributed to variations in immune responses to particular strains of virus. Further research is needed to elucidate the immune mechanisms associated with BVDV infections in alpacas.

As with post-natal cattle exposed to BVDV, the alpacas in this current study were observed to undergo transient immunosuppression, as evidenced by decreased total leukocyte counts, even in the absence of clinical signs of disease (Potgieter, 1995). The difference in the mean pre-inoculation WBC count and the mean nadir WBC count reflected a 59% (Group1), 56% (Group 2), and 44% (Group 3) decrease in total WBC counts. This percentage of decrease is similar to trends seen in cattle intranasally exposed to BVDV (Roth et al., 1981). In cattle, acutely infected animals remain important not only due to the immunosuppressive and direct effects of the virus on the host, but also the potential for this animal to shed BVDV to other animals (Thurmond, 2005). In cattle, there is evidence that acutely infected animals may be able to maintain the virus within a population (Smith & Grotelueschen, 2004). The acutely infected animal may be epidemiologically important within the alpaca industry as travel and

cohabitation are required for breeding management, shows, and social gatherings. Of course, any existing PI crias remaining with their dam during these times would also disseminate BVDV.

It has been proposed that close proximity and population dynamics could allow interspecific transmission of a pestivirus (Nettleton, 1990). This concept has been evidenced by serological studies that revealed a great number of New World camelids being seropositive when in close proximity to cattle or goats (Belknap et al., 2000; Wentz et al., 2003; Mattson et al., 2006). Serological evidence also exists for heterologous infection. A survey in Norway revealed that 4.5% of sheep and 2.2% of pigs possessed high antibody titers to BVDV (Loken et al., 1991b) and Graham et al discovered a 30.4% seroprevalence to BVDV in sheep herds in Ireland (Graham et al., 2001). Antibodies to BVDV were found in 4.3% of unvaccinated captive exotic ruminants (Doyle & Heuschele, 1983). Bison in North America were found to possess a 31% seroprevalence for BVDV antibodies (Taylor et al., 1997). Around 2% of 390 llamas in Argentina tested positive for virus neutralizing antibodies to BVDV (Puntel et al., 1999). The seroprevalence rate for llamas tested in Oregon was 4.4% of 270 sampled; more than half of these positive animals were from a farm where llamas grazed with cattle (Picton, 1993). An 11% seroprevalence for BVDV antibodies was identified in alpacas in Peru that had been cohabitated with sheep and cattle (Rivera et al., 1987). Wentz reported a seroprevalence of 0.9% in llamas and alpacas in North America (Wentz et al., 2003). These studies demonstrate exposure and subsequent seroconversion to BVDV in heterologous species.

Intraspecific transmission may be occurring in alpacas (Evermann, 2006; Topliff et al., 2009). Suggestively, to date, BVDV 1b remains the most predominant genotype isolated from alpacas (Byers et al., 2009; Carman et al., 2005; Foster et al., 2005; Foster et al., 2007; Goyal et al., 2002) and herds with PI cria exposure have documented seroconversion in adults (Carman et al., 2005; Topliff et al., 2009). In a new study in the U.S.A., sequencing data from 46 BVDV isolates from alpacas placed all isolates as BVDV 1b. Of the 46 isolates, 45 shared greater than 99% nucleotide identity in the 290 base pair 5′ untranslated region. The exception, a Canadian isolate, shared 96.5% homology. This new data points toward maintenance of a unique genotype of BVDV 1b in the alpaca population (Kim et al., 2009). Kim et. al. give two theories for the presence of this phylogenetically unique BVDV 1b in alpacas. One theory is that mere chance resulted in a BVDV 1b PI cria, which propogated the disease through movement and contact with naïve alpaca herds. The second theory suggests that only unique BVDV 1b genotypes can accomplish transplacental infections to result in a PI cria (Kim et al., 2009). A recent BVDV prevalence study of 63 alpaca herds in the USA found 4 crias with confirmed persistent infections (Topliff et al., 2009). Of the four BVDV isolates obtained from PI herds, three were found to have close homology (96.4-97.2%) with NY-1 (a BVDV 1b) and 1 was found to have close homology (97.6%) with NADL (a BVDV 1a) (Topliff et al., 2009). This study remains to be the only study that indicates infection of alpacas with BVDV 1a, while other literature continues to confirm the presence of only BVDV 1b in alpacas. In addition, this concluded that both the presence of PIs and the use of bovine and caprine colostrum contributed to the number of seropositive animals in a herd (Topliff et al., 2009). Based upon our experiment and the literature to

date, it appears that both interspecies and intraspecies transmission may play potential roles in the epidemiology and ecology of BVDV in alpacas.

Based upon results from this current study, our recommendations for implementation of a BVDV biosecurity and wellness plan for alpacas would include several management principles. First, a quarantine period should be established for animals that have been exposed to any animals (cattle or alpacas) of unknown BVDV status. Based upon detection of nasal shedding of virus for up to 14 days post-exposure in this study, this quarantine period should be at least 21 days. Secondly, stress should be minimized for up to 12 days after a possible exposure to BVDV. During this time, acutely infected alpacas will be immunosuppressed and will likely exhibit a greater susceptibility to opportunistic infections. Wherever possible, producers should maintain a closed herd, reduce comingling of alpacas, and reduce intimate contact of alpacas and cattle.

In conclusion, the strain of BVDV isolated from alpacas (genotype 1b) caused the slowest onset of viremia when compared to the BVDV strains of bovine origin, and was slowest in causing a nadir in white blood cell counts. It is unknown whether this is due to biological replication of the specific strain or titer of viral inoculation. However, the isolation of virus from white blood cells in all study groups indicates active viral replication. Furthermore, all three evaluated strains induced nasal shedding of infectious virus particles. With the exception of one animal in the BVDV 2 cattle exposure group, all animals in the study seroconverted, and animals exposed to the BVDV 1b alpaca strain had the highest titers on day 28 post-inoculation between study groups. To our knowledge, this is the first published report to characterize the results of acute infection

of alpacas with BVDV genotypes 1b and 2 of cattle origin and a BVDV 1b of alpaca origin.

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APPENDICES

APPENDIX A

Red top tubes for Serum collection Processing:

1. Centrifuge at 200 x g (1100rpm) for 20 min
2. Collect serum into a sterile Sarstedt tube (2ml) under the hood
3. Store in -80°C freezer

APPENDIX B

Buffy Coat Processing:

Purple top EDTA tubes for WBC collection

1. Aliquot 10 ml of 0.15 M NH_4Cl in a 15-ml centrifuge tube, keep at 4°C
2. Centrifuge at 700 x g (2020 rpm) 4°C for 30 min. The centrifuge in room 104 Sugg lab, program 9
3. Using a sterile Pasteur pipette carefully extract the white blood cells band and place it in a 15-ml centrifuge tube with 10 ml of 0.15 M NH_4Cl, mix well
4. Centrifuge at 700 x g (2020 rpm) 4°C for 10 min
5. Pour off the supernatant
6. Add 10 ml of MEM media [1.1] and mix well, centrifuge at 700 xg (2020 rpm) 4°C for 10 min
7. Pour off the supernatant
8. Resuspend the WBC pellet in 0.5 ml of MEM media

Do not freeze WBC. Setup VI test [4.1] fresh or passage [2.2]

For alpaca WBC samples: after step 3 keep tubes in refrigerator for 1 hr

0.15 M NH_4Cl

Ammonium Chloride: Sigma cat. #A5666-500G
FW 53.49

8.02 gm (53.49 x 0.15 = 8.02) NH_4Cl in 1000 ml of Millipore H_2O, mix well and filter through 0.22µm vacuum filter. Aliquot 10 ml of 0.15 M NH_4Cl in a 15-ml centrifuge tube, keep at 4°C

APPENDIX C

IMMUNO-PEROXIDASE ASSAY FOR NONCYTOPATHIC BVDV

1. Reagents to be prepared for the immunoperoxidase assay include:

    a) Fixative for fixation of cells prior to assay.

    b) Diluent for preparation of monoclonal antibodies and conjugate

    c) Washing mixture for steps to remove unbound antibodies.

    d) Preservation media for preserving stained cells.

1. Reagents can be prepared in advance and stored at 4° C for several weeks.

FIXATIVE		DILUENT	
Saline (0.85%)	**80.00 ml**	**D-PBS**	**100.00 ml**
BSA (1%)	20 µl	BSA (1%)	20 µl
Acetone	20.00 ml	Tween 20	50 µl
Total Volume	**100 ml**	**Total Volume**	**100 ml**

WASH MEDIA PRESERVATION MEDIA

D-PBS	**500.00 ml**	**D-PBS**	**96.0 ml**
Tween 20	0.25 ml	Formaldehyde	4.0 ml
Total Volume	500 ml	Total Volume	100 ml

(D-PBS: GIBCO-BRL Life Technologies catalog # 14040133)
(0.85% saline is mixed in the lab with 8.5 g NaCl/1000 ml Millipore water)
(BSA:

2. Preparation of monoclonal antibodies working solutions; this assay utilizes a mixture of two separate anti-BVDV monoclonal antibodies:

   a). D89 from Dr. Harish Minocha, Kansas State is diluted 1:500 in diluent
   (10 µl: 5000 µl) is enough for one 96-well plate

   b). 20.10.6 from Dr. Ed Dubovi, Cornell Univ. New, York is diluted 1:800 in diluent
   (10 µl:8000 µl) is enough for 1 ½ 96-well plates.

3. Preparation of conjugated rabbit anti-mouse IgG antibody: a working solution is diluted 1:150 by mixing 40 µl conjugate in 6000 µl diluent. The source for this reagent is Jackson Immuno-Research catalog # 315-035-0030.

5. Madin Darby bovine kidney (MDBK) cells, determined to be free of BVDV are used
   for culture of the virus in this assay. Cells are grown in monolayers

   propagated in 25 cm2 flasks. Cells are maintained in culture medium

composed of Minimum essential medium (MEM) supplemented with 10 % equine serum (v/v), 1% L-Glutamine (29.2mg/ml), 1% NaHCO3 (75mg/ml), and 1% Pen/Strep/Fungizone (10,000Units/10,000µg/25µg/ml).

ASSAY AND TITRATION PROCEDURES

1. A 96-well cell culture plate is inoculated with sample(s) to be tested by one of two methods:
   a) add 90 µl of MEM w/ 10% EQS to each well and add 10 µl of test sample and make serial dilutions down the plate by carrying 10 µl from well to well.
   b) add 50-100 µl of undiluted test sample to each of three wells.

2. A confluent monolayer of MDBK cells is washed two times with 5 ml of Ca 2+ and Mg 2+ free PBS and trypsinized with 1 ml of trypsin-EDTA for 2 minutes. The activity of the trypsin is stopped by adding 11 ml of culture medium. The mixture is vigorously pipetted to create a single cell suspension and transferred to a sterile trough.

3. With the aid of a multi-channel pipettor, 50 µl of suspended cells are placed in each inoculated well of the 96-well culture plate. Each plate should also contain a positive and a negative control. The plates are then incubated for 72 hours at 38.5 °C and 5% CO_2.

IMMUNOPEROXIDASE TEST

1. After incubation, medium is dumped from all wells and the plate is air dried under the hood. It is critical to allow the cells to dry thoroughly (at least one hour) otherwise detatchment of cell monolayers could occur during the following steps.

2. One hundred microliters of fixative are dispensed into each well and fixation is allowed for 10 minutes at room temperature. After this time, as much fixative as possible is discarded by dumping into the sink followed by tapping inverted plates on a double layer of paper towels. Plates are again allowed to air dry for at least one hour (at this point the plates could be preserved at -20° C for a few weeks if the assay could not be finished immediately).

3. Diluted anti-BVDV antibody (50 µl per antibody per well) is added to each well and incubated for 20 minutes at 37° C. (Antibody mixture contains 10 µl D89/5 ml diluent and 10 µl 20.10.6/8 ml diluent for each plate, these are mixed in separate tubes).

4. Plates are washed 3 times with PBS-T, to remove unbound antibody, by adding 100 µl of washing mixture to each well with the aid of a multichannel pippettor. Plates are gently rocked or tapped by hand and the wash is discarded into the sink followed by tapping inverted plates on a double layer of paper towels. As much as possible of the liquid is discarded.

5. Fifty microliters of diluted, conjugated rabbit anti-mouse IgG is dispensed per well and again incubated for 20 minutes at 37° C. (40µl conjugate/6 ml diluent is 1:150)

6. Plates are washed again with 100 µl per well as described in step 4.

7. Substrate is prepared immediately before use according to the manufacturer's (ZYMED INC. AEC Substrate Kit) instructions as follows:

a) Measure 5 ml millipore water/plate in a plastic snap cap tube.
b) Add 5 drops of reagent A, Substrate Buffer (20X) to the water and mix.
c) Add 5 drops of reagent B, AEC Chromogen (20X) to the above and mix.
d) Add 5 drops of reagent C,0.6% Hydrogen Peroxide (20X) and mix well.
e) Dispense 50 µl per well and incubate 15 minutes at room temperature.

8. Substrate is replaced with 100 µl of preservation media per well and read immediately using a light microscope. Observe positive and negative controls first. A reddish-brown stained cell is considered positive and the lack of any color is interpreted as negative.

9. When wrapped in aluminum foil, plates can be stored in the refrigerator at 4° C for several days to weeks. The color reaction will fade over time.

APPENDIX D

Passages of Serum, WBC and Nasal swab samples to best detect acute infections for Dr. Jason Johnson 2007-1237 study

1. All samples will be passed and assayed by using **BTU** cells,

2. All samples will be passed as described below.

    a. Seed 9.6 cm^2 wells of a 6 well plate (BD Falcon® 353046) with **BTU** cells by adding 250 µL of cell suspension (from 25 cm^2 flask in 12 mL of media) to 2.5 mL of MEM + 10% EQS.

    b. Incubate the plate in the clean incubator for 24 hours.

    c. Observe cells to make sure each well was seeded.

    d. Label wells with sample identification to be added.

    e. Remove old media.

    f. **Serum** Passage: Add 192 µL of fresh MEM + 10% EQS to each well, add 768 µL of serum from each sample, total volume 960 µl

    g. **WBC** passage: Add 960 µl of WBC

    h. **Nasal Swab** Passage: Add 960 µl of Nasal Swab

    i. Incubate for 1 hour.

    j. After incubation add 3 mL of MEM + 10% EQS to each well. Be very careful to avoid cross-contamination.

    k. Incubate the 6-well plate for 4 days before wrapping the plate in aluminum foil and freezing at -80°C.

    l. When the plate is thawed, pipet the samples up and down 3 times to break up any cells and place cell suspension in 2.0 mL Sarstedt tubes.

3. Assay by standard immunoperoxidase monolayer assay using **BTU** cells, a 3-day incubation and dilution of 10 µL of cell suspension into 90 µL of media.

APPENDIX E

Virus Neutralization Test Protocol

1. Heat inactivate the test sera for 30 min at 56ºC.
2. Add 50 µL of MEM-eq medium to all wells of a cell culture grade flat-bottomed 96-well microtitre plate. Add 50 µL of heat-inactivated serum to the top row of the plate. From a starting dilution of 1:2, make serial two-fold dilutions of test sera, using the MEM-eq medium as diluent. For each sample, three wells are used at each dilution. Dilutions are made such that 50 µL remain in each well for each dilution.
3. To each well, add 50 µL of stock virus diluted in MEM-eq such that the inoculum contains 4 $CCID_{50}$/µL (a total of 200 $CCID_{50}$/50 µl). A back titration (1:10) of virus stock is also done in some spare wells to check the potency of the virus used for each virus neutralization protocol.
4. Incubate for one hour at 38.5 ºC in 5%CO_2.
5. Trypsinize a 25 cm^2 flask of MDBK cells. Add 50 µL of cell suspension to each well of the microtitre plate.
6. Incubate the plate in 5%CO_2 for three days at 38.5ºC.
7. Dump the plate and perform immunoperoxidase staining for the noncytopathic viruses used for VN in this lab.[4.1.2]
   After 24 hrs of incubation start checking CPE for the cytopathic viruses[4.1.4].
8. Simplified endpoint determination—greatest dilution at which 1 of 3 wells are free of virus.

APPENDIX F

Clinical Scoring Chart

Experimental exposure of naïve alpacas to Bovine Viral Diarrhea Virus 1b, 1b-alp, and 2.

Group _____

Date: _____

Animal ID						
Appearance						
Appetite						
Attitude						
Fecal score						
Nasal Discharge						
Initials of Observer						

Appearance:
0= Normal mentation, bright, alert, responsive
1= Inactive, depressed, will move or get up with encouragement
2= Recumbent, depressed, will not move even with encouragement

Appetite:
0= Normal, eating fine
1= Decreased appetite, limited interest in eating
2= Not eating

Attitude:
0= Normal stance, good posture, head held erect
1= Awkward stance, ambulates awkwardly
2= No regular stance, unable to ambulate

Fecal Score:
0= No abnormal feces noted
1= Watery, loose diarrhea
2= Bloody, loose diarrhea

Nasal Discharge:
0= No discharge present
1= Small amount clear mucus
2= Copious amount, colored