

The Pennsylvania State University
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**A PHYLOGENETIC ANALYSIS OF
BOVINE VIRAL DIARRHEA VIRUS
SUBTYPES IN DIAGNOSTIC SAMPLES
FROM CATTLE IN PENNSYLVANIA**

A Thesis in

Pathobiology

by

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ABSTRACT

Bovine Viral Diarrhea (BVD) is an economically important disease in cattle with estimated losses between 10 and 40 million dollars per million calvings per year in the US. BVD is a complex viral disease producing multifocal clinical symptoms. Evidence from previous studies of Bovine Viral Diarrhea Virus (BVDV) in the United States has shown that 1 in 133 BVDV isolates were similar to strains of BVDV routinely used in vaccinations. The purpose of this study was to: 1. Analyze clinical BVDV isolates obtained from samples submitted to the Pennsylvania State University Animal Diagnostic Laboratory for genetic similarity to BVDV vaccine strain subtype. 2. To determine if novel strains and subtypes of BVDV are circulating in vaccinated and unvaccinated cattle and to make inferences about the efficacy of the current vaccines. The 5 prime untranslated region (5' UTR) of the BVDV genome from BVDV was isolated from clinical samples by reverse transcription polymerase chain reaction (RT-PCR). PCR products were purified and sequenced in both directions using forward and reverse primers. The resulting sequences were compared to known BVDV reference strains representing 10 subtypes of BVDV currently described worldwide. Phylogenetic analysis was used to group isolates based on similarity to known BVDV subtypes. The conclusions of this study describe 5 subtypes of BVDV identified in the study population. The majority of isolates were found to be dissimilar to BVDV strains found in common vaccines for BVDV. An unexpectedly high rate of diversity of BVDV 2b was found in the study population and a diverging phylogenetic grouping from the BVDV 2b was identified as a possible new subtype of BVDV in cattle.

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Chapter 1

LITTERATURE REVIEW

Introduction and Background

1.1. Clinical features and types of BVDV infection

Bovine Viral Diarrhea Virus (BVDV) is a Flavivirus belonging to the Pestivirus genus. All BVD viruses can be categorized into two genotypes. BVDV type 1 is more commonly occurring than BVDV type 2. Severe acute infections associated with BVDV 2 may cause death in animals 10-24 hours after first symptoms are noticed (Houe, 2003b). BVDV infection with either genotype can be either acute or persistent in individual animals. Acute infections are also known as Transient Infections (TI) and occur after the animal is born and may last days to weeks. Animals can build immunity to BVDV during transient infections and effectively clear the virus and recover from the infection. The main reproductive symptom of BVD is early termination of pregnancy. Other developmental issues are associated with BVD infections as well such as low birth weight, fever, loss of appetite and failure to thrive (Ridpath *et al.*, 2000). Persistent infection (PI) differs from TI in that PIs may last the lifetime of the animal and may not cause noticeable symptoms of disease. PIs occur when a calf is born after BVDV has passed from the dam to the calf during pregnancy. Calves persistently infected with BVDV generally have a lower rate of weight gain as well as an overall lower weight at the time of weaning.

BVDV may show a variety of clinical signs of illness. BVDV infection may be acute or subclinical. Cattle with acute infections may exhibit: fever, leukopenia, depression, anorexia and lower milk production. Severe acute infections may result in respiratory distress, pneumonia and thrombocytopenia, bloody diarrhea, and hemorrhagic symptoms. Other livestock species such as alpaca and deer are susceptible, and develop similar clinical signs to BVDV infections as in cattle.

BVDV infections can lead to the establishment of Mucosal Disease (MD) in PI animals. MD occurs when a PI animal infected with a non-cytopathic strain of BVDV is subsequently

infected and develops an acute infection with a cytopathic (or antigenically different) strain of BVDV (Sentsui *et al.*, 2001). MD is characterized by severe clinical signs usually accompanied by progressive dehydration leading to death within 3 to 10 days. Mortality rates in cattle with MD usually approach 100% (Brownlie & Clarke, 1993; Goyal & Ridpath, 2005).

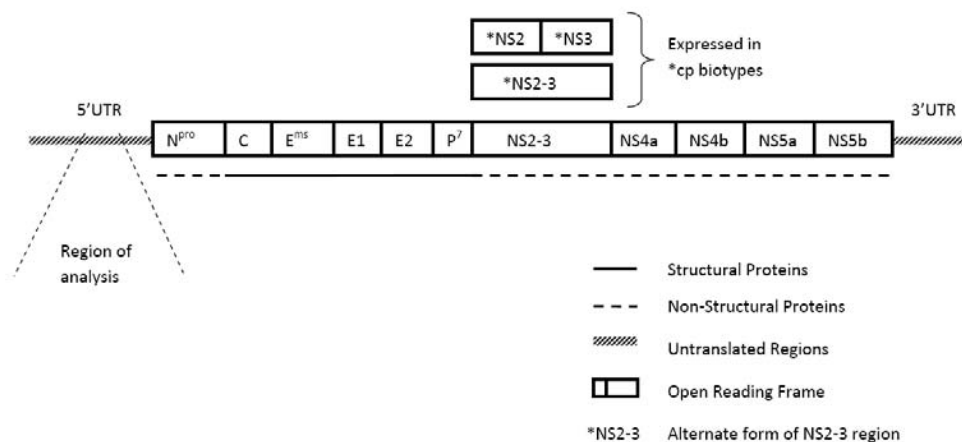
MD may arise from subsequent infection from spontaneous mutation of the established BVDV strain, infected herd members or from inoculation with modified live virus vaccine (Ridpath & Bolin, 1995).

BVDV is a problem for cattle producers because it is difficult to detect and causes significant economic losses at the herd level. BVDV is a challenge to control in herd animals because of the establishment of PI individuals. PI animals continuously shed large amounts of virus particles in body fluids while showing no clinical signs of infection. A single PI animal can repeatedly expose a whole herd to BVDV virus over a long period of time. In addition to the presence of large numbers of viral particles shed by PI animals, BVDV is effectively spread through aerosol transmission to the nasal mucosa. Direct nose to nose contact is thought to be the most common and effective route of transmission. BVDV has also been shown to spread through freshly contaminated pens (Niskanen & Lindberg, 2003). Direct costs to the herd owner associated with BVDV include the cost of diagnostic testing, vaccine purchase, high abortion rate, and animal disposal (Larson *et al.*, 2005). Indirect costs include losses due to slow weight gain in calves and low milk production. Overall poor herd health may be attributed to BVDV and may be associated with increased susceptibilities to other viral and bacterial diseases (Tiwari *et al.*, 2009). As of July of 2009, 41.4 million cattle were calved in the United States (USDA NASS report on July 2009 for US cattle) with an estimated losses of 10 to 40 million dollars per million calvings per year in the US (Houe, 2003a). The total financial loss due to BVDV in the US as of July of 2009 was calculated at between 414 million and 1,656 million dollars by using these data references.

1.2. BVDV general genomic features

The genome is comprised of a linear single stranded positive sense RNA molecule. The entire BVDV genome is about 12,500 bases (12.5 Kb) in length. At the five prime end there is a 381 nucleotide 5 prime untranslated region (5'UTR) and a 229 nucleotide untranslated region at the three prime end (3'UTR). The 5' and 3' UTR flank a single open reading frame (ORF) of 11,700 nucleotides, which makes up the majority of this Pestivirus genome (Collett *et al.*, 1988). and encodes 12 protein regions, including 5 structural proteins and 7 non-structural polypeptides (Figure 1.1). An alternate form of the NS2-3 nonstructural polypeptide region is found in cytopathogenic (cp) biotypes of BVDV. Most of The 5'UTR is well conserved among Pestivirus species with 86% to 93% homology among all BVDV 1 genotypes and a greater than 90% homology to known BVDV 2 genotype viruses (Kim & Dubovi, 2003). For this reason the 5'UTR has been used extensively in molecular assays for specific detection and characterization of BVDV genotype and subtype analysis.

*Figure 1.1. Map of the BVDV genome. The alternate form of the NS2-3 region is noted here by showing the NS2-3 region individually and the alternate translation of the region as individual nonstructural polypeptide regions NS2 and NS3 (Quadros *et al.*, 2006).*



Like other RNA viruses, the BVDV virus is subject to a high rate of mutation of its nucleic acid due to an error prone polymerase. There is about 1 mutation in every 10 Kb replicated in BVDV which translates to one mutation for every complete genome replication. Mutations in RNA viruses are usually caused by the misincorporation of a nucleotide or point mutation at a random position. Point mutations can have different outcomes for the virus. Some mutations may give rise to a more virulent form of the parent virus while others may change the host specificity or tissue affinity of the virus. Alternatively, mutations may result in a lethal outcome or a non viable virus (Hamers *et al.*, 2001).

Changes in the BVDV genome caused by mutations have given rise to a diversity of BVDV viruses within the cattle and alpaca populations in Pennsylvania (Vilcek *et al.*, 2001). Detection and examination of virus diversity at the nucleotide level may reveal patterns of virus diversity that may allow more successful vaccine production strategies (Kalaycioglu, 2007).

1.3. Laboratory diagnosis of BVDV

BVDV is difficult to detect in herd situations because the virus manifests in BVD syndrome that causes a wide range of clinical signs with varying degrees of severity. BVDV may go undetected for long periods of time in a herd of cattle if clinical signs are not severe and routine diagnostic testing is not part of herd management (Houe & Meyling, 1991). The major threat to herd health caused by BVDV is the presence of PI animals in the herd. PI animals may shed large amounts of BVDV throughout their lifetime (Houe, 2003b). The only way to reliably detect PI animals is through laboratory testing for BVDV (Sandvik, 2007). PI cattle are born with BVDV. When a cow is infected with a non-cytopathic form of BVDV between 90 and 120 days of gestation the fetus may become infected and tolerant to the BVDV. This is because the immune system of the fetus is not fully formed at the time the virus infects its body tissue. BVDV disseminates throughout the organs of the fetus during development. When immunocompetence

develops in the fetus, BVDV is not recognized as foreign and no immune response is mounted (McClurkin *et al.*, 1984).

Diagnostic assays to determine herd status may be performed by ear notch tests for BVDV using Immunohistochemistry (IHC) or antigen capture ELISA testing to detect antigen. The ear notch test is performed on a small tissue sample that has been cut away from the ear of an animal and is embedded in paraffin wax and sequentially sliced into microscopic sections using histological techniques. The small sections are then stained using immunohistochemistry (IHC) techniques and examined under a microscope for the presence of BVDV by detection of chromophores that have attached to the BVDV viral particles (Brodersen, 2004). Diagnostic tests on serum using Enzyme-linked Immunosorbent Assay (ELISA) are also used to detect BVDV using antibodies for BVDV specific virus proteins. A common ELISA test for BVDV is Antigen capture ELISA (ACE) A popular herd screening test is the use of Real Time RT-PCR to detect BVDV in pooled serum samples. In this test, several individual serum aliquots are combined in a pool. RNA is then extracted from each pool and is used as template in a Real Time PCR tests using PCR to detect the 5' UTR of the BVDV genome. If a serum pool is determined to be positive using this method the individual serum samples included in the pool are tested individually to discover which of the animals is infected with BVDV (Bhudevi & Weinstock, 2001).

The availability of rapid and accurate laboratory tests to detect BVDV is convenient for the quick dissemination of disease information for veterinarians and herd health decision makers. ACE and PCR based tests provide a highly accurate and sensitive means to detect BVDV. Information regarding virus characteristics and growth patterns may be attained using Virus Isolation (VI) procedures. Results from VI testing require more time than ELISA or PCR based assays but VI testing provides an absolute conclusion of the presence of the virus. Many

diagnosticians use a combination of laboratory testing procedures to confirm the presence of BVDV in laboratory samples before making a final diagnosis.

Recently, a study showed specificity and sensitivity of several diagnostic tests for BVDV. The study reported accuracy of the tests by sample types as determined by correctly detected negative or positive non-pooled samples. Known positive or negative samples were submitted to 23 laboratories following recommended submission procedures for IHC (skin), ACE (skin), PCR (serum) and VI (serum). The results were summarized in Table 1-1. The conclusion of the study showed ACE tests on skin samples were 100% accurate for correctly reporting known positive or negative results. VI testing produced the least accurate results according to the study (Edmondson *et al.*, 2007). Pooled samples are often used in laboratory testing for BVDV and are used to improve throughput and lower the cost of herd testing. However test sensitivities are affected by pooling samples of any type. Serum and whole blood samples are sample types that are the most commonly pooled sample type for laboratory detection of BVDV (Gaede *et al.*, 2003; Saliki *et al.*, 2000).

Table 1-1 Percentage of accurate diagnostic test results from known status samples submitted to 23 diagnostic laboratories for detection of BVDV (Edmondson et al., 2007)

IHC (skin)		ACE (skin)		VI (serum)		PCR (serum)	
%CP	%CN	%CP	%CN	%CP	%CN	%CP	%CN
90	98	100	100	69	98	85	89

CP= Correct Positive, CN= Correct Negative

1.4. Genotypes of BVDV

BVDV is commonly classified into two genotypes BVDV 1 and BVDV 2. Viruses from BVDV 2 are primarily associated with severe acute BVDV (Pellerin *et al.*, 1994; Ridpath *et al.*,

1994). Each genotype of BVDV may contain the cytopathogenic biotype or the non-cytopathic biotype. Genotypes of BVDV are determined by nucleic acid characterization while biotypes of BVDV are determined by cytopathogenicity of the strain. It is thought that actual occurrence of BVDV 1 may be generally underestimated in the cattle industry due to subclinical BVD1 infections (Evermann & Ridpath, 2002).

BVDV 1 and BVDV 2 are distinguished as different BVDV genotypes associated with differing severity of disease. The genotype of BVDV that is more often associated with severe clinical symptoms and outbreak potential is BVDV 2 (Ridpath *et al.*, 2000). A large outbreak of BVDV among veal calves in the Great Lakes region of North America led to the identification of BVDV 2 in 1994 (Ridpath *et al.*, 2006). Characterization of the BVDV virus genome has classically been performed by sequence analysis in any of three regions of the BVDV genome. These regions are the 5' UTR (Vilcek *et al.*, 2001), non-structural N-terminal protein (Npro) region and the non-structural protein two and three (NS2/3) region (Flores *et al.*, 2002; Tajima *et al.*, 2001). The Npro and the NS2/3 region are in the ORF of the genome and both of these regions are highly conserved within the BVDV genome. However, the 5'UTR is considered most the most highly conserved region of pestiviruses (Deng & Brock, 1993). There is good agreement in genotypic classification when using any of these regions and all of these regions have been used at one time or another to characterize the BVDV virus at the subgenotype level (Kim *et al.*, 2009; Vilcek *et al.*, 2001). Studies involving the characterization of the NS2/3 region at the nucleotide level have shown that the NS2/3 region is associated with BVDV biotype determination while the 5'UTR is not (Collett *et al.*, 1989). BVDV genotyping has been used on a regular basis in laboratory diagnostic testing. PCR techniques provide a fast and reliable means of detection. Several different RT-PCR assays have been developed for the detection of BVD. Real Time RT-PCR and conventional RT-PCR have been used to detect

BVDV in several diagnostic sample types with reliable results. (Kim & Dubovi, 2003; Letellier & Kerkhofs, 2003; Rossmanith *et al.*, 2001)

Nucleotide analysis of the 5'UTR region of the BVDV genome reveals that BVDV can be subdivided beyond the genotype level of classification. Differentiation of distinct groups within the BVDV 1 genotype has been shown to result in two main subtypes: BVDV 1a and BVDV 1b (Ridpath & Bolin, 1998). More recent research has shown that BVDV 1 can be subdivided into 11 subtypes (Vilcek *et al.*, 2001). Studies have shown that BVDV subtype 1b is present in the cattle population in the United States and Canada at various levels and has been shown to be the predominant subtype of BVDV in alpaca (Kim *et al.*, 2009). BVDV 2 has been shown to contain at least 2 subtypes: BVD2a, BVD2b (Flores *et al.*, 2002).

Subtypes of BVDV have been associated with the host species in which they were first discovered (Vilcek & Nettleton, 2006). For example the BVDV strain found to infect giraffe is known as the Giraffe strain of BVDV and so on (Becher *et al.*, 1997). The giraffe strain of BVDV has been used as a reference in many phylogenetic studies but typically is not associated with outbreaks in cattle or alpaca. Strains of BVDV associated with deer have also been included in many phylogenetic studies. Deer may serve as a natural reservoir for BVDV (Van Campen *et al.*, 2001). Studies involving surveillance of BVDV in deer have been performed to explore this concern (Brooks *et al.*, 2007). The establishment of specific strains of BVDV in host species other than cattle shows the widespread distribution and evolutionary adaptability of pestiviruses such as BVDV. Swine and sheep are known carriers of closely related pestiviruses Classic Swine Fever (CSF) and Border Disease. The genome of the Border Disease virus (BDV) is nearly genetically identical to BVD 2 (Becher *et al.*, 1998). It has been argued whether the viruses are the same (Dinter & Morein, 1990). The major reservoir for BVDV is presently known as PI cattle. However, BDV has been shown to be vertically transmitted in sheep resulting in persistent infection (Nettleton *et al.*, 1992) PI BVDV has been shown in alpaca (Carman *et al.*, 2005;

Mattson *et al.*, 2006) and in white-tailed deer (Passler *et al.*, 2007). Recently, BVDV PI mousedeer in a Danish zoo transmitted BVDV subtype 1f to bovine calves when exposed in experimentally a typical pen situation (Uttenthal *et al.*, 2005). This particular strain was shown to vary only by a few nucleotides from strains of BVDV 1f found in German cattle (Tajima *et al.*, 2001).

1.5. Biotypes of BVD

BVDV may or may not exhibit cytopathic effect on infected cells in culture, and cytopathic (cp) and non-cytopathic (ncp) biotypes may exist within each specific BVDV subtype and while the cytopathogenicity of a strain has obvious outcomes in cell culture the same is not always true within the host. The biotype of a BVDV strain may have a role in the progression of disease within the host and has been shown to play a role in the transmission of the virus to the fetus during pregnancy (Bielanski *et al.*, 2009). Cytopathogenicity of the BVDV infecting the host plays an important role in pathogenesis. PI calves are only produced during pregnancy and only when infected with ncp strains of BVDV (Radostits, 1986; Roeder *et al.*, 1986). Acute clinical signs of MD arise when a PI animal either contracts a cp form of BVDV or develops a homologous cp form of BVDV (Becher *et al.*, 2001; Bolin *et al.*, 1985b). PI cattle may also develop MD after vaccination with modified live virus vaccine containing a cp form of either BVDV 1 or BVDV 2 (Ridpath & Bolin, 1995). Post-vaccination MD does not always arise from administration of cp strains in Modified Live Vaccines (MLV) to PI animals (Bolin *et al.*, 1985a). In cases where the introduced cp strain is antigenically different from the PI ncp strain host antibodies form against the introduced strain allowing the PI animal to clear the virus (Fulton *et al.*, 2003).

Cytopathogenicity of strains can be studied with nucleotide scale analysis of the BVDV genome. The factors that play a role in cytopathogenicity have been attributed to the NS2/3

region of the BVDV genome. The 5'UTR and the NS2/3 regions have been used to determine subtype on the nucleotide level with good agreement between both regions (Vilcek *et al.*, 2001). However the 5'UTR has not been shown to be directly responsible for cytopathogenic properties of the virus (Fritzemeier *et al.*, 1995; Fritzemeier *et al.*, 1997).

PI animals pose the largest challenge to BVDV management in herds. PI animals with ncp BVDV strains usually develop MD when additionally infected with a cp BVDV strain. The super-infection of cp BVDV may be introduced from common routes of disease transmission (usually contact with infected animals) or may arise due to recombination of the ncp strain with an introduced cp strain.

In an experiment in 1993, Moenning and others compared a cp strain recovered from cattle with MD to cp strains experimentally administered 1 month earlier. The re-isolated strain was analyzed for similarity to the inoculated cp strain and was found to be a recombination of the cp strain administered and the initial infecting ncp strain. A specific monoclonal antibody comparison for the expressed viral protein E2 found in the ncp strain was performed. The antibody reactivity pattern of the re-isolated cp strain matched the pattern of the persisting ncp strain. Genomic sequence comparisons of both strains showed the re-isolated strain as having sequence homology consistent with the cp configuration of the NS2/3 region. Thus the re-isolated cp strain expressed the ncp form of the E2 protein while possessing the cp form of the NS2/3 region (Moennig *et al.*, 1993). The same occurrence has been observed after the administration of MLV vaccine containing cp strains in PI cattle (Ridpath & Bolin, 1995).

1.6. Aspects of vaccination

Vaccination for BVD in cattle is commonly practiced. Many different types of vaccines are available. Herd inoculation is often performed by herd managers or owner/operators on advice from a professional veterinarian. Vaccines for BVDV are typically sold as vaccine cocktails

containing modified live virus or killed virus containing many different strains of BVDV viruses or BVDV in combination with other viruses. Many of the virus vaccines available contain vaccine components against common bovine viruses such as: BVDV, Parainfluenza Virus type 3 (PI3), Infectious Bovine Rhinotracheitis (IBR) and Bovine Respiratory Syncytial Virus (BRSV) (Bergeron & Elsener, 2008). BVDV strains that are used in vaccines in the US are primarily composed of BVDV subtype 1a cytopathic strains (Fulton *et al.*, 2003; Goyal & Ridpath, 2005). Modified live virus (MLV) and killed vaccine virus strains are available that contain strains of both BVDV 1 and BVDV 2 but subtype data for all vaccines available is not generally published by vaccine manufacturers. Table 1-2 shows commercially available BVDV vaccines and strain information. Recent studies show the BVDV 1b is the predominant subtype of BVDV found in both cattle and alpaca (Fulton *et al.*, 2002; Kim *et al.*, 2009).

Table 1-2. Strains and types of BVDV used in vaccines (Fulton et al., 2003)

Vaccine Type	Name of Strain	Subtype/Biotype	Manufacturer
MLV			
Arsenal 4.1	GL 760	1 ncp ¹	Novartis
Express 5	Singer 296	1a cp 2a cp ²	Boehringer Ingelheim Vet Medica
BoviShield 4	NADL	1a cp	Pfizer Animal Health
Pyramid 4	Singer	1a cp	Fort Dodge Animal Health
Reliant 4	NADL	1a cp	Merial
Frontier 4 Plus	C24V 296	1a cp 2a cp ²	Intervet
Titanium 5	C24V 296	1a cp 2a cp ²	Agrilabs
Jincine 4	WRL	1 ncp	Schering-Plough Animal Health
Herd Vac 3	Singer	1a cp	Biocor Animal Health
Bovine Viral Diarrhea Vaccine	C24V	1a cp	Colorado Serum Co.
Killed			
Elite 4	Singer	1a cp	Boehringer Ingelheim Vet Medica
Horizon 4 Plus	C24 V 125C	1a cp 2a cp	Intervet
Master Guard 5	C24 V 125C	1a cp 2a cp	Agrilabs
Respishield 4	Singer	1a cp	Merial
Triangle 4 + type II	Singer 5912	1a cp 2a cp	Fort Dodge Animal Health
CattleMaster 4	5960 6309	1a cp 1 ncp	Pfizer Animal Health
ViraShield 5	KY22 TN 131	1a cp 2 ncp	Grand Laboratories
Surround 4	Singer NY-1	1a cp 1b ncp	Biocor Animal Health

cp = cytopathic; ncp = noncytopathic

¹Information provided by manufacturer

²Based on sequencing information by Dr. J. F. Ridpath, USDA, ARS, NADC

Two types of vaccines are available for BVDV, Modified Live Virus (MLV) vaccine and killed virus vaccine. MLV types of vaccines differ in that live viruses in MLV replicate in the host to produce an immunogenic response while viruses found in the killed vaccine are non-replicating and produce a host response targeted directly against killed virus. MLV vaccines require smaller initial volumes at the time of vaccination due to the active replication of the live virus within the host. MLV vaccines are advertised as providing lifelong immunity to BVDV with a single inoculation. Killed virus vaccines usually require a larger initial volume at the time of vaccination. Vaccine manufacturers recommend that a second inoculation is administered at 140 days after initial inoculation for killed vaccines (Talens *et al.*, 1989). Antibodies to both BVDV 1 and BVDV 2 have been shown to circulate for up to 180 days after inoculation with a MLV vaccine (Cortese *et al.*, 1998). Other Studies have shown circulating antibodies for BVDV 1 and BVDV 2 have been detectable up to 18 months after vaccine inoculation. Circulation of vaccine derived viruses may complicate the diagnosis of BVDV by laboratory testing. After administration of MLV vaccine, BVDV viremia may occur for 3 to 7 days. Viruses isolated from animals that have been administered MLV vaccine within a two week time period should be differentiated from vaccine strains of BVDV when diagnosis of disease is performed (Fulton *et al.*, 2003). BVDV 1 and BVDV 2 are antigenically different from each other in serum neutralization assays using polyclonal and monoclonal antibodies (Ridpath *et al.*, 2000). While vaccines containing BVDV 1a are able to induce antibodies against BVDV 2 the efficacy of the cross reactivity may not be adequate enough to provide protection against all strains and subtypes of BVDV 2. This is demonstrated in the occurrence of BVDV 2 PI calves born from dams that have been vaccinated for BVDV 1. (Bolin *et al.*, 1991; Ridpath *et al.*, 1994) Antigenic variation exists between BVDV 1a and BVDV 1b (Ridpath & Bolin, 1998). These two subtypes are distinguishable by monoclonal antibody binding patterns (Vilcek *et al.*, 2000). Immunologic protection against BVDV 1b from vaccines that contain only BVDV 1a strains have been tested

in experimental vaccination studies with mixed results. A killed vaccine containing BVDV 1a cp strain along with a 1a ncp strain were used in a vaccine challenge with BVDV 1b ncp (NY-1) as the challenge virus. Incomplete protection was found against BVDV1b in this study (Talens *et al.*, 1989). An earlier experimental vaccination was performed using a MLV vaccine containing BVDV 1a NADL cp strain was performed on day 27 using BVD 1b ncp (NY-1) as the challenge virus. None of the vaccinated cattle showed clinical signs of illness after 14 days (Phillips *et al.*, 1975).

Table 1-2 shows 18 BVDV vaccines along with the BVDV content of killed virus and modified live virus for BVDV. Seventeen of the 18 vaccines contain viruses of the BVDV 1a subtype and one contains the BVDV 1b subtype. No killed virus vaccine contains strains of BVDV 1b. Thirteen of the 18 vaccines contain only cytopathic forms of BVDV. The most often used strain of BVDV found in these vaccines is the Singer BVDV 1a strain followed by the C24V 1a strain. Antigenic cross reactivity of these strains have been established by several vaccine immunogenicity studies in which antibody titers have been measured against several strains of BVDV 1 and BVDV 2 (Cortese *et al.*, 1998; Fulton *et al.*, 1995; Fulton *et al.*, 1997). These studies have been performed using vaccines that contain BVDV 1a cp strains almost exclusively. Many of these studies do not include BVDV subtype descriptions for vaccine strains, field strains or challenge strains (Goyal & Ridpath, 2005). Information regarding BVDV strain subtype content in vaccines is generally not available from manufacturers in neither vaccine label nor vaccine trial literature. Information used for subtype definitions in this study were obtained entirely from academic literature. Information regarding cytopathogenicity of strains contained in vaccines is generally reported by vaccine manufacturers in both vaccine labels and vaccine trial literature. Presumably this allows the end user to determine the appropriate timing of vaccination regarding breeding schedule.

The use of MLV vaccine carries tradeoffs in herd health with a risk of post vaccination disease within the herd. MLV vaccine administered to pregnant cattle has been shown to cause abortion and development defects (Liess *et al.*, 1984; Orban *et al.*, 1983). Due to these abortions and developmental defects described in the study the authors question the use of MLV in BVD vaccination. Sweden is using such a national control program for BVDV that includes the goal of total eradication of BVDV (Greiser-Wilke *et al.*, 2003; Stahl *et al.*, 2005). In and 1980 experiment by Done *et al.* MLV vaccines containing cp strains of BVDV were administered to pregnant cattle in the third trimester of pregnancy. Calves that were born from the dams were positive for BVDV and only ncp strains of BVDV were recovered from the calves (Done *et al.*, 1980). This suggests that significant virus mutation and recombination occurred in the dams post-vaccination.

Vaccination against BVDV in cattle herd management is commonly practiced to control BVDV. Recent studies of vaccine efficacy and show that current vaccines do not provide immunity against commonly circulating subtypes of BVDV in the United States (Fulton *et al.*, 2003; Fulton *et al.*, 2006a; Ridpath, 2005; Talens *et al.*, 1989). Vaccine manufacturers rely on cross reactive antigenic immunity induced by vaccine strains of BVDV subtype 1a as a means of protecting cattle from other subtypes that are known to circulate in the cattle population in the US (Flores *et al.*, 2000; Pellerin *et al.*, 1994; Wolfmeyer *et al.*, 1997). While cross reactivity has been shown to be established against many subtypes of BVDV other than 1a the induction of effective immunity provided by the vaccines are in question in current literature. (Arenhart *et al.*, 2008; Fulton *et al.*, 2005b; Fulton *et al.*, 2006b; Fulton *et al.*, 2007; Fulton *et al.*, 2009) Data from several studies indicate that cross reactive antigenic immunity provided by both MLV and killed vaccines containing BVDV 1a are inadequate against BVDV 1b infections in herd exposures to PI BVDV 1b (Pellerin *et al.*, 1994; Talens *et al.*, 1989). One study established that even a killed vaccine containing BVDV 1b was ineffective at inducing immunity against BVDV 1b when an

experimental herd was exposed to PI animals infected with BVDV 1b (Fulton *et al.*, 2005a). This particular study suggests that the timing and rate of seroconversion may indicate that etiological patterns of infection by BVDV 1b may differ from etiological patterns of BVDV 1a, thus changing the efficacy of the vaccine against BVDV 1b. The goal of this study is to characterize BVDV strains recovered from cattle at the subgenotype level and look for patterns that may indicate inadequate vaccination coverage for some subtypes of BVDV from cattle in Pennsylvania with the specific aim of describing the distribution of BVDV subtypes in vaccinated and non-vaccinated cattle. Analysis of the distribution of subtypes in each study group will be used to determine if current vaccination programs provide incomplete protection against some BVDV subtypes. The hypothesis of this study is that: Subtypes of BVDV found to occur equally in both vaccinated and unvaccinated cattle reveal that vaccines used against BVDV in Pennsylvania do not provide adequate protection against some subtypes of BVDV. Additionally this study will show the diversity of BVDV subtypes that occur in Pennsylvania cattle.

Chapter 2

MATERIALS AND METHODS

2.1. Samples

Serum and pooled tissue samples from 109 cattle were obtained from bovine submissions to the Pennsylvania Animal Diagnostic Laboratory System (PADLS) between 1997 and 2009 with requests for BVDV testing by RT-PCR or Virus Isolation. All blood samples were centrifuged at 2000 r.p.m. for 10 minutes using a refrigerated tabletop Beckman Centrifuge, Model GS-6R. (Beckman-Coulter, Inc Fullerton, CA) Serum was removed and stored until further testing. All blood samples were obtained from diagnostic herd screening; serum pools of 30 individuals per pool were compiled by removing 100 uL from the individual serum after centrifugation. 400 uL of the resulting pooled sera were used for BVDV detection by RT-PCR. Tissue samples were processed for RNA extraction and RT-PCR or Virus Isolation according to PADLS Standard Operating Procedures. All tissues and cell culture materials from positive samples were archived and stored at -80°C.

2.2 Identification of Study Groups

BVDV positive serum and tissue samples were selected according to BVDV vaccination status and the presence or absence of clinical signs of the animal of origin. Vaccination status and clinical history was obtained by examination of case submission documentation using information from USALIMS (Laboratory Information Management System) telephone survey of herd owners or archival sample submission documents at Pennsylvania State University Animal Diagnostic Laboratory (ADL). Below is an outline of the structure of the survey. The telephone survey was conducted by:

1. Personal introduction and purpose of the call.
2. Establishing recollection of the specific case and animal or animals in question.
3. Specific questions about vaccination history and clinical signs of the animals involved.

Two specific questions were asked during each call;

Question 1: Were the animals sick or did they show signs of weakness or respiratory distress at the time that veterinary samples were taken?

Question 2: Have the animal or animals been previously vaccinated for BVD?

The results of the survey were tabulated and study groups were established according to the responses from the herd owners.

Table 2-1. Numbers of samples identified in each of 4 study groups.

Study Groups	Clinical Signs	No Clinical Signs	Totals
Previously Vaccinated	35	22	57
Not Previously Vaccinated	38	14	52
Totals	73	36	109

2.3. RNA isolation

RNA was extracted from serum samples (400 uL) and cell free supernatant using the MagMax AI/ND Viral RNA Isolation Kit (Ambion, AM1929, Foster City CA.) and eluted in 40uL nuclease free water (30ng/uL). RNA was also extracted from tissue samples (500 mg) using the Trizol extraction method (Invitrogen catalog number 6096020, Carlsbad CA). The resulting RNA pellet was eluted in 250uL diethylpyrocarbonate (DEPC) treated water to a final concentration of 1ug/ul. RNA from positive serum and tissues were stored at -80°C.

2.4. Sequencing known positive BVDV samples amplified by RT-PCR

The segment of the BVDV genome used to characterize each virus was a 290 nucleotide portion of the 5'UTR. The PCR primer pairs used were described according to the site of binding when using the NADL reference strain of BVDV (GenBank accession number M31182). The primer sequences were designed to amplify a region of the 5'UTR common in BVDV viruses (Weinstock *et al.*, 2001). The forward primer bound at nucleotides 104-124 while the reverse primer bound at nucleotides 372-392. The resulting PCR fragment was 289 nucleotides in length. The primer sequences are described in Table 2-2.

Table 2-2. Oligonucleotide primers for partial amplification of the 5' UTR of BVDV viruses.

Name	Nucleotide Sequence	NADL BVDV 1 strain nucleotide position
Forward Primer	5'- TAGCCATGCCCTTAGTAGGAC - 3'	104-124
Reverse Primer	5' - ACTCCATGTGCCATGTACAGC - 3'	392-372

Each 50uL RT-PCR reaction was performed in triplicate in the 96 well plate format using Invitrogen Super Script One Step RT-PCR with Platinum Taq (Invitrogen; 10928-042, Carlsbad CA). Each 50 uL RT-PCR reaction was prepared using 0.2 uM Pestivirus Forward and Reverse PCR primers. 100ng RNA template was added to each sample. RT-PCR reactions were performed in triplicate. RNA was reverse transcribed at 50°C for 30 minutes followed by deactivation of reverse transcriptase at 95°C. cDNA was amplified by 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds a final hold for 10 minutes at 72°C was

followed by an infinite hold at 4°C. The RT-PCR reactions were performed using an Applied Biosystems Gene Amp 9600. (Applied Biosystems, Foster City, CA)

Table 2-3. BVDV Reference strains for phylogenetic analysis using the 5'UTR.

	Subtype	Name	Accession	Reference Source	Designator
1	1a	NADL	M31182	(Colett, Larson et al. 1988)	1a NADL M31182
2	1a	Singer	DQ088995	(Jones, Zandomeni et al. 2006)	1a Singer DQ088995
3	1b	Libra	FJ387288	(Kim, Anderson et al. 2009)	1b Alpaca Libra FJ387288
4	1b	Orion	FJ387299	(Kim, Anderson et al. 2009)	1b Alpaca Orion FJ387299
5	1b	NY-1	FJ387232	(Kim, Anderson et al. 2009)	1b NY-1 FJ387232
6	1b	Osloss	M96687	(Demoerlooze, Lecomte et al. 1993)	1b Osloss M96687
7	1c	CH692	AY671983	(Pizarro-Lucero, Celedon et al. 2006)	1c CH692 AY671983
8	1c	Deer	AB040132	(Harasawa,R. 2007)	Deer AB040132
9	1c	Deer 544	EU597009	(Pogranichniy, Raizman et al. 2008)	Deer 544 EU597009
10	1d	B397-06	EU224234	(Hornberg, Fernandez et al. 2009)	1d B397-06 EU224234
11	1d	H-AT	FJ493478	(La Rocca and Sandvik 2009)	1d H-AT FJ493478
12	1e	8-Fr	FJ493479	(La Rocca and Sandvik 2009)	1e 8-Fr FJ493479
13	1e	B50-05	EU224244	(Hornberg, Fernandez et al. 2009)	1e B50-05 EU224244
14	1f	1562F	FJ493481	(La Rocca and Sandvik 2009)	1f F 1562 FJ493481
15	1f	JA-TF	FJ493480	(La Rocca and Sandvik 2009)	1f J A-T FJ493480
16	1g	A-AT	FJ493482	(La Rocca and Sandvik 2009)	1g A ATF J493482
17	1g	L-AT	FJ493483	(La Rocca and Sandvik 2009)	1g L ATF J493483
18	1i	23-13	FJ493484	(La Rocca and Sandvik 2009)	1i 23-13 FJ493484
19	1i	23-15	FJ493485	(La Rocca and Sandvik 2009)	1i 23-15 FJ493485
20	2a	1373	AF145967	(Ridpath, Bendfeldt et al. 2006)	2a 1373 AF145967
21	2a	890	U18059	(Ridpath 2005)	2a 890 U18059
22	2b	51966	EU371402	(Mishra, Rajukumar et al. 2008)	2b India-51966 EU371402
23	2b	Soldan	U94914	(Canal, Strasser et al. 1998)	2b Soldan U94914

2.5. Sequence Alignment and Phylogenetic Analysis

PCR fragments were purified by spin column (Qiagen, Valencia, CA) and submitted to Davis Sequencing Inc. in Davis CA for sequencing. Analysis was performed using reverse primers on purified fragments (5.8 ng/uL) using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) Sequence data were retrieved using the Davis Sequencing web service and imported to EditSeq software, part of the DNASTAR Lasergene 7.1 software package (DNASTAR Inc., Madison, WI). In some cases, the reference sequences contained nucleotide information for the entire genome. ClustalW multiple sequence alignment was performed only on the 289 nucleotide region of the BVDV genome that corresponded with nucleotide positions 104-392 described for the NADL strain of BVDV (GenBank accession number M31182).

A series of phylogenetic trees were constructed to show genomic relationships and prevalence of strains within the study groups. The phylogenetic trees that were generated contain both reference sequences of known BVDV subtypes and unknown field strains. The length of each branch on a node of the phylogenetic tree represents the number of nucleotide replacements the alignment software performed for optimal pair wise alignment in MegAlign Software (DNASTAR Inc., Madison, WI). Sequence alignment was performed using the ClustalW method in MegAlign software. (DNASTAR Inc., Madison, WI) Reference sequences representing known subtypes of BVDV were imported into MegAlign along with the sequences obtained from field virus strains. Each BVDV subtype was represented by two reference sequences.

Reference sequences for BVDV subtypes were obtained from the NCBI website (National Center for Biotechnology Information, Bethesda, MD: National Library of Medicine, US). Sequences were selected from literature to represent known subtypes of BVDV virus. (Table 2-3) Many of the reference sequences were in the form of whole genome sequences. Reference strains were chosen by according to which subtype they represented within the BVDV genotypes.

Chapter 3

RESULTS AND DISCUSSION

3.1. PCR results

Sixty- one of the 109 archived samples did not produce PCR products. A breakdown of the BVDV isolates resulting in an amplified fragment from the 5'UTR RT-PCR reaction are denoted in Table 3-1.

Table 3-1. Number of isolates per group that were submitted for sequencing.

Study Groups	Clinical Signs	No Clinical Signs	Total
Previously Vaccinated	20	10	30
Not Previously Vaccinated	14	4	18
Total	34	14	48

3.2. Results from phylogenetic analysis of Pennsylvania field strains

Twenty five of the BVDV isolates from diagnostic cases, regardless of study group, were identified as subtypes of BVDV 1 and share close sequence homology with the reference subtypes of BVDV 1b (n=21). Subtypes for BVDV 1a (n=1), and BVDV 1c (n=3) were also found. Twenty three of the isolates were subtypes of BVDV 2. The only cluster diverging from the BVDV1b appeared to be herd specific but still closely related to BVDV 1b and did not represent a new subtype, Figure 3-1. Two field strains were shown to share sequence homology with BVDV reference subtypes that were derived from Whitetail Deer in the United States fitting into the subtype category of BVDV 1c (n=3). No field isolates were found to share significant similarity to any of the following BVDV 1 subtypes: 1d, 1e, 1f, 1g, 1i. Results indicated that the only subtypes of BVDV that were present in the samples submitted to ADL between 1997 and

2009 are 1a, 1b, 1c, 2a, and 2b. In light of these findings the subtypes 1d, 1e, 1f, 1g and 1i will not be shown in calculations and comparisons in subsequent analysis.

Two subtypes of BVDV genotype 2 were identified; BVDV 2a (n=9) and BVDV 2b (n=14). A relatively large portion of the BVDV 2b branching arrangement contained several sequences that significantly diverged from the main branching for BVDV 2b. This divergent grouping of BVDV 2b subjects will be referred to as BVDV 2b* (n=7). Currently only two subtypes of BVDV 2 are described worldwide. A summary of the individuals in the phylogenetic analysis is shown in Table 3-2.

Figure 3-1. Phylogenetic tree of field strains as compared to reference strains. Nodes: N1, similarity shown between the majority of field strains with subtype BVDV 1b; N2, herd specific subtype of BVDV 1b; N3, field isolates share similarity with subtype BVDV 1c and BVDV subtypes isolated from deer; N4, BVDV 2b subtypes; N5, BVDV 2a subtypes; N6, field strains showing divergence from known BVDV 2b subtypes.

Figure 3.1

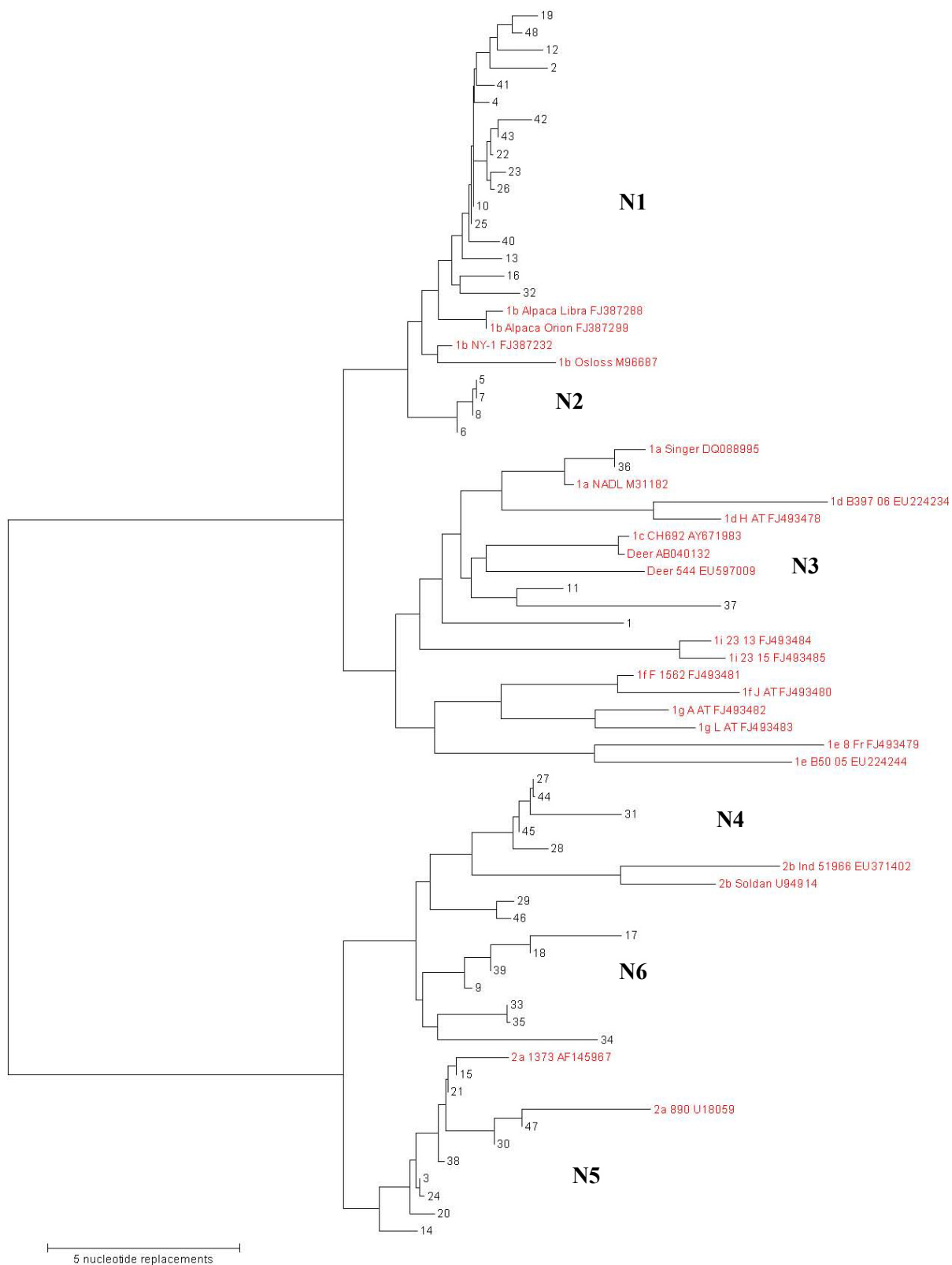


Table 3-2. Summary of BVDV subtypes in Pennsylvania cattle from 1997-2009. Subtypes in each study group by percent and number of field strains, Group 1, not vaccinated with no clinical signs (V-/CS-), Group 2, vaccinated with no clinical signs of illness (V+/CS-), Group 3, not vaccinated with clinical signs of illness (V-/CS+), Group 4, vaccinated with clinical signs (V+/CS+). Number of individuals (n) noted in parentheses next to percentage for each subtype.

group	1a	1b	1c	2a	2b	total
1	. (0)	50.0% (2)	25.0% (1)	25.0% (1)	. (0)	4
2	. (0)	77.8% (7)	11.1% (1)	. (1)	10.0% (1)	10
3	. (0)	42.9% (6)	. (0)	28.6% (4)	28.6% (4)	14
4	5.0% (1)	30.0% (6)	5.0% (1)	15.0% (3)	45.0% (9)	20
total	2.1% (1)	43.8% (21)	6.3% (3)	18.8% (9)	29.2% (14)	48

3.3. Results for Study Groups

Results for the number of subtypes found in each study group were summarized and tested using Chi square analysis. The summaries are the percentages for individuals making up each study group (Table 3-2). Data show that subtype BVD 1b is equally distributed among study groups regardless of vaccination or clinical signs $\chi^2(3,n=21)=.422$ $p>.05$. Data also show that BVDV 2b is not evenly distributed in study groups $\chi^2(3,n=14)=.002$ $p>.05$. BVDV 2b was found with greatest frequency in vaccinated cattle showing clinical signs of illness (n=9).

3.4. Results from sequence alignments

Sequence alignments were performed using pair wise alignment to further define the relationship of the individual sequences that comprised node N6 and for N4 in Figure 3-1 (data are shown in Figure 3-2 and 3-5). A consensus sequence was constructed using ClustalW to represent the individuals that made up node N6 and N4 respectively and used in the comparison. The BVDV 1a NADL strain (M31182) was used as a nucleotide position reference. BVDV 2a

strain 890 and 2b Soldan (U18059, U94914) were included in the comparison. The consensus sequences for each node were compared to BVDV 2a and 2b reference sequence to confirm the isolate identity by pair wise alignment and to confirm percent similarity between sequences.

Figure 3-2. Sequence alignment of isolates that comprised node N6 of the phylogenetic tree for the study population. Numbers in the left hand column refer to the individual sequences corresponding to the sequence labels in N6 of the phylogenetic tree. Labels: NADL 2a, 2b and Consensus, describe reference strains and the consensus sequence for N6.

```

CLUSTAL 2.0.12 multiple sequence alignment

18      --TTAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 58
17      --TTAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 58
39      --TTAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 58
Consensus
9      ---TAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 57
35      -TTAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 59
33      -TTAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 59
2a      GGCTAGCCATGCCCTTAGTAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 60
2b      -----TAGGACTAGCAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 42
NADL    ---TAGCCATGCCCTTAGTAGGACTAGCAT AATGAGGGGGTAGCAACAGTGGTGAATTC 57
          ***** ** ***** ** ***** ** ***** *

18      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 118
17      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 118
39      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 118
Consensus
9      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 117
35      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAGTT 119
33      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAGTT 119
2a      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 120
2b      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 102
NADL    GTTGGATGGCCCTTAGTACAGGGAAGTCGTCAATGGTTCGACACTCCATCAATC 116
          ***** ** ***** ***** ***** ***** ***** *

18      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 178
17      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 178
39      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 178
Consensus
9      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 179
35      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 179
33      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 179
2a      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 180
2b      AGAGAGCTCGAGATGCCATGTGGACGAGGGCATGCCACGGCACATCTTAACTATGCG 162
NADL    AAG--GTCTCGAGATGCCACGTGGACGAGGGCATGCCAAAGCACATCTTAACTATGCG 174
          * ***** ***** ***** ***** ***** ***** *

18      GGGGTTGCATGGGTGAAAGC-GCCAT-TCGTAGCGTTATGGACACAGC----- 224
17      GGGGTTGCATGGGTGAAAGC-GCCAT-TCGTAGCGTTATGGACACAGCTTAGACT---- 232
39      GGGGTTGCATGGG-GAAAGC-GCCAT-TCGTGG----- 208
Consensus
9      GGGGTTGCATGGGTGAAAGC-GCCAT-TCGTAGCGTTATGGACACAGCCTGATAGGGTGT 237
35      GGGGTTGCATGGGTGAAAGC-GCCAT-TCGTAGCGTTATGGACACAGCCTGATAGGGTGT 235
33      GGGGTTGCATGGGTGAAAGC-GCCAT-TCGTGGTGTATGAGCACAGCCTGATAGGGTGT 237
2a      GGGGTTGCGTGGGTGAAAGC-GCCAT-TCGTGGTGTATGAGCACAGCCTGATAGGGTGT 237
2b      GGGGTTGCGTGGGTGAAAGC-GCCAT-TCGTGGTGTATGAGCACAGCCTGATAGGGTGT 238
NADL    GGGGTCGCCAGGTAAAAGCAGTTTTTAAACCGACTGTTACGAATACAGCCTGATAGGGTGC 224
          ***** ** ** ***** *

18      -----
17      -----
39      -----
Consensus
9      AGCAGAGACCTG----- 249
35      AGCAGAGACCT----- 246
33      AGCAGAGACCTG----- 249
2a      AGCAGAGACCTGC----- 250
2b      AGCAGAGACCTG----- 251
NADL    AGCAGAGACCTGCTATTCGCTAGTATAAAAACTCTGCT----- 261
          TGCAGAGGCCACTGATTGCTACTAAAAATCTCTGCTGTACATGGCACATGGAGT 290

```

Figure 3-3. Sequence alignment of isolates that comprised node N4 of the phylogenetic tree for the study population. Numbers in the left hand column refer to the individual sequences corresponding to the sequence labels in N4 of the phylogenetic tree. Labels: NADL 2a, 2b and Consensus, describe reference strains and the consensus sequence for N4.

```

CLUSTAL 2.0.12 multiple sequence alignment

Consensus_N4      TTTTAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 60
45                TTTTAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 60
44                TTTTAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 60
27                ---TAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 57
31                --TTAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 58
28                --TTAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGG--ACTAGCGGTAGCAGTGAGTTC 57
2a_890           GGCTAGCCATGCCCTTAGTAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 60
2b_sol1dan      -----TAGGACTAGCAAAAGGAGGGGACTAGCGGTAGCAGTGAGTTC 42
1a_NADL         ---TAGCCATGCCCTTAGTAGGACTAGCAT AATGAGGGGGGTAGCAACAGTGGTGAGTTC 57
                    ***** ** ***** ***** ** ***** *

Consensus_N4      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCACCAATC 120
45                ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCACCAATC 120
44                ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCACCAATC 120
27                ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCACCAATC 117
31                ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAAAGGTTCAACACTCCACTAATC 118
28                ATTGGATGGCCGAATCCATGAGTACAGCGAAGTCGTCAATGGTTCGACACTCCACCAATC 117
2a_890           ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACACTCCCTTTAGTC 120
2b_sol1dan      ATTGGATGGCCGAATCCCTGAGTACAGGGAAGTCGTCAATGGTTCGACGCTCTGGCAACA 102
1a_NADL         GTTGGATGGCTTAAGCCCTGAGTACAGGGTAGTCGTCAATGGTTCGACGCTTGG--AATA 116
                    ***** ** ** ***** ** ***** ** ***** ***** ** *

Consensus_N4      GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 180
45                GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 180
44                GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 180
27                GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 177
31                GAGGAGTCTTGAGATGCCCTGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 178
28                GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCC--ACGGCACATCTTAACTATGCG 176
2a_890           GAGGAGTCTCGAGATGCCATGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 180
2b_sol1dan      AGAGAGCTCTCGAGATGCCATGTGGACGAGGGCATGCCCCACGGCACATCTTAACTATGCG 162
1a_NADL         AAG--GTCTCGAGATGCCACGTGGACGAGGGCATGCCCCAAAGCACATCTTAACTATGAGCG 174
                    * ** ***** ***** ***** * ***** ***** *****

Consensus_N4      GGGGTTGCATGGGTGAAAGCACCAT--TTGTGG--TGTTATGGACACAGCCTGATAGGGTGT 238
45                GGGGTTGCATGGGTGAAAGCACCAT--TTGTGG--TGTTATGGACACAGCCTGATAGGG-- 235
44                GGGGTTGCATGGGTGAAAGCACCAT--TTGTGG--TGTTATGGACACAGCCTGATAGGGTGT 238
27                GGGGTTGCATGGGTGAAAGCACCAT--TTGTGG--TGTTATGGACACAGCCTGATAGGGTGT 235
31                GGGGTTGCATGGGTGAAAGCACCAT--TTGTGG--TGTTATGGACACAGCCTGATAGGGTGT 236
28                GGG--TTGCATGGGTGAAAGCACCAT--T--GTGG--TGTTATGGACACAGCCTGATAGGGTGT 232
2a_890           GGGGTTGCGTGGGTGAAAGCGCCAT--TCGTGG--CGTCATGGACACAGCCTGATAGGGTGT 238
2b_sol1dan      GGGGTTGCGTGGGTGAAAGCATCATCTTGTGA--TGTTATGGACACAGCCTGATAGGGTGT 221
1a_NADL         GGGGTCGCCAGGTAAAAGCAGTTTAAACCGACTGTTACGAATACAGCCTGATAGGGTGC 234
                    ***** ** ** ***** ** ***** ** ***** ***** *****

Consensus_N4      AGCAGAGACCTGC----- 251
45                ----- 251
44                AGCAGAGACCTGC----- 251
27                AGCAGAGACCTG----- 247
31                AGCAGAGACCTGC----- 249
28                AGCAGAGACCC----- 243
2a_890           AGCAGAGACCTGC----- 251
2b_sol1dan      AGCAGAGACCTGCTATCCGCTAGTATAAAAACTCTGCT----- 261
1a_NADL         TGCAGAGGCCACTGTATTGCTACTAAAAATCTCTGCTGTACATGGCACATGGAGT 290

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3.5. Discussion

This study shows for the first time the spectrum of BVDV subtype diversity in vaccinated and non-vaccinated cattle within the study population. Many of these subtypes have only recently been described as occurring in small populations of cattle from distant geographic locations. Subtypes of BVDV 1d, 1e, 1f, 1g, 1i were not found in the study population and their overall incidence of these mentioned subtypes is currently unknown.

Table 3-2 shows the frequency of subtypes for all study groups. BVDV 1a had the lowest frequency of all of the subtypes (n=1). The next lowest frequency was subtype 1c (n=3). The reference strain used to define subtype 1a in this study was the Singer strain of BVDV. The Singer strain of BVDV has been historically chosen by vaccine manufactures for use in cattle vaccines for BVDV because of properties of cross reactivity with other types of BVDV viruses (Reber *et al.*, 2006; Tiwari *et al.*, 2009). Either the Singer strain or the NADL strains are found in the majority of available vaccines and vaccines containing these strains protect against ncp strains of BVDV genotype 1 and not protecting against cp strains of BVDV genotype 1 (Fulton *et al.*, 2002). The reference strain of BVDV 1c used in this study shares a 94% sequence homology with BVDV 1a Singer strain in the 5'UTR region (BLAST, data not shown). The data from this study have shown that subtypes 1a and 1c are virtually absent from the study population regardless of vaccination status or clinical signs. This suggests that the population of cattle studied is displaying classic herd immunity for BVDV subtypes 1a and closely associated strains. Herd immunity in epidemiology is defined as a type of immunity that occurs when the vaccination of a portion of the population (or herd) provides protection to unprotected individuals (John & Samuel, 2000). These data may be interpreted to show that subtypes of BVDV associated with deer may be kept in check because of herd immunity in cattle for BVDV viruses that share a high sequence homology. This evidence may contribute to the findings presented in the article published by Brooks showed the absence of BVDV virus in necropsied farm raised

deer in Pennsylvania in 2004. (Brooks *et al.*, 2007) The data found in the present study along with the findings of Brooks et al. support the theory that herd immunity resulting in low occurrence of BVDV subtype 1a and 1c may be taking place in BVDV vector species in Pennsylvania.

Our data demonstrate that BVDV 1b is equally represented in all study groups regardless of vaccination status or presentation of clinical signs and is the most frequently occurring subtype in the total study population Table 3-2. These two factors combined showed that BVDV vaccination in Pennsylvania between the years 1997 and 2009 may have provided inadequate immunologic protection against BVDV subtype 1b, 2a and 2b. Evidence of the establishment BVDV 1b infections in a population of vaccinated cattle can be found in a study performed at the University of Wisconsin, Madison by Talens and others in 1989. The study used killed vaccine strains BVDV 1a cp, and BVDV 1 ncp (subtype not mentioned) administered in two doses (day 1 and day 14). Antibodies for BVDV were measured and protection from illness was established against a non-vaccinated control population. A vaccine challenge using BVDV 1b (NY-1) was performed on day 28. Antibody titers of the vaccinated population were between 1:16 and 1:128 (mean = 1:42.7). Protection against the challenge strain was shown to be incomplete because illness occurred in a portion of the vaccinated population. (Talens *et al.*, 1989)

A study published in 2009 of a phylogenetic analysis of BVDV subtypes found in Alpaca from 8 states in the United States and Canada showed that 100% (n=46) of Alpaca tested at the Cornell University Animal Health Diagnostic Center were of the 1b subtype (Kim *et al.*, 2009). BVDV 1b made up a total of 91% of all BVDV 1 infections in our study. BVDV 1b was responsible for 43.75% of overall incidence of BVDV when all subtypes of BVDV 1 and BVDV 2 are considered. Host specificity of BVDV 1b must be taken into consideration with the findings of both studies. Kim and Anderson suggest that Alpaca may be preferentially susceptible to BVDV 1b through species specificity (Kim *et al.*, 2009). The results of this study

indicate that currently available vaccines for BVDV may provide inadequate protection against BVDV 1b.

An article published in the September 2009, stated that there were signs of bovine viral diarrhea virus (BVDV), even after the observed cattle had been vaccinated for BVDV several times previously. BVDV 1b was detected in cultures taken from the cattle by veterinarians. The herd veterinarian concluded BVDV 1b was circulating in cattle showing clinical signs of illness even though the cattle that had been vaccinated more than once with commercial vaccines containing BVDV 1a and BVDV 2a (Ishmael 2009). The article addressed vaccination of cattle herds in the Midwestern United States. The cattle herds of Pennsylvania presumably are vaccinated using the same pool of commercially available vaccines. Many of those vaccines are specifically mentioned in this study. The thrust of the article is that commercial vaccines are regarded as being ineffective against BVDV 1b. The data and finding of this study reinforce this hypothesis.

Data from this study show an unexpected genetic diversity of BVDV 2 subtypes in the study population. Recent literature describes the occurrence of BVDV 2b in the United States as relatively rare in North America in comparison to BVDV 2a. (Goyal & Ridpath, 2005) Although the data set presented in this study is relatively small (n=48) the overall percentage occurrence of BVDV 2b (29%, n=14) exceeds the occurrence of BVDV 2a (18.5%, n=9). Furthermore this pattern of BVDV 2 subtype occurrence appears similar to patterns reported for South America by Flores and others in 2002 where the occurrence of BVDV subtype 2a and 2b were detected at similar levels of occurrence. (Flores *et al.*, 2002)

A phylogenetic group diverging from the reference strains for BVDV 2b used in this study was observed. BLAST results showed that previous isolates of BVDV 2 found in goats share a high rate of homology with the isolates that make up the divergent phylogenetic grouping from this study (N6 in Figure 3-1). The isolates that showed the greatest homology were

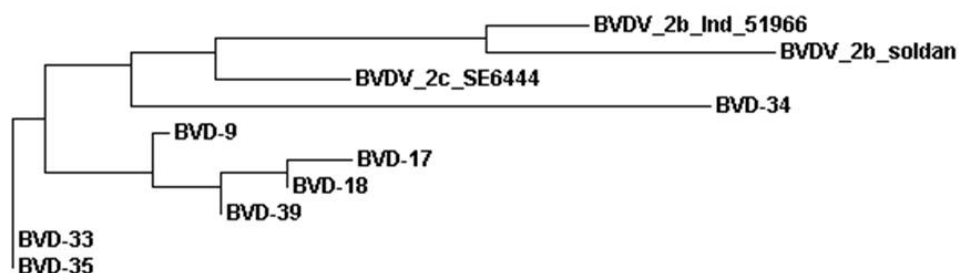
submitted to NCBI GenBank by the Cornell University Animal Health Diagnostic Center. (Kim and Anderson 2008, unpublished). Criteria for defining a new BVDV subtype are subject to debate. The establishment of subtype 2b by Flores (n=4) and others showed a 18% sequence divergence from BVDV 2a strains to establish subtype BVDV 2b using the 5' UTR and 13% using the NS2/3 region. (Flores *et al.*, 2002) This study shows that isolates that comprised the N6 node of the phylogenetic tree showed a 5% sequence divergence from BVDV 2a strain 890 and 7% divergence from BVDV 2b Soldan strain. Vilcek and others have suggested that South American isolates of subtype BVDV 2b and the North American isolates of subtype BVDV 2a have a range of divergence between 19% and 1% between subgenotypes in the 5' UTR (Vilcek *et al.*, 2004). The consensus sequence from isolates that comprised N6 was 22% divergent from the BVDV 1a NADL reference sequence.

Results from the overall phylogenetic comparison of field strains and references subtypes show the emergence of a cluster of BVDV genotype 2 field strains that diverge in similarity from BVDV 2b subtypes using the partial sequence of the BVDV 5'UTR. Sequence alignment reveals that the isolates that make up this grouping contain genomic elements from both BVDV 2a and BVDV 2b. Initial phylogenetic analysis of the entire study population shows that this grouping is divergent from both subtype 2a and 2b but sequence alignment shows that these isolates share slightly less divergence from the BVDV 2a reference strain (5%) than the BVDV 2b reference strain used in this study (7%). Further classification of these isolates would have to be performed to quantify the sequence similarity with other Pestiviruses and possibly define a previously unknown subtype of BVDV 2.

In an additional analysis a BVDV isolate identified as BVDV 2c (Beer *et al.*, 2002) was used as a reference strain and compared to the isolates that made up N6 and reference strains for BVDV 2b used in Figure 3.1. A sequence alignment was performed and a phylogenetic tree was constructed using the methods mentioned above (Figure 3-4). This analysis of the previously

described BVDV 2c isolate showed a greater similarity to reference strains for BVDV 2b than to isolates that made up node N6 in Figure 3-1.

Figure 3-4. Phylogenetic tree showing isolates from node N6 and reference strains for BVDV 2b and BVDV 2c.



Both killed and modified live vaccines contain strains of BVDV 2a and not 2b.

Recombination events may take place within an animal after receiving a modified live virus vaccine containing the BVDV 2a strain. This study shows that both BVDV 2a and BVDV 2b were recovered from animals who received vaccination for BVDV. Specific information regarding the exact vaccine type was not available but one can assume that BVDV 2b was not introduced by vaccination. Recombination events in the inoculated animal may explain the presence of BVDV 2b in the study population. However the exact criteria for defining subtypes BVDV 2a and 2b remain in question. More information is required regarding the delineation of BVDV 2 subtypes in order to conclude that isolates of N6 comprise an unknown subtype. However if BVDV 2b is present in the study population then it must be considered a new finding. In that BVDV 2a is described in current literature as by far the more rare subtype of BVDV 2 present in North America. (Fulton *et al.*, 2005b; Kim *et al.*, 2009; Vilcek *et al.*, 2004) If the findings of this study are correct then BVDV 2b must be considered a candidate as an emerging virus in the United States. The N4 grouping of the phylogenetic tree appears closely related to the BVDV 2b reference strains. To investigate the validity of the N4 node of the phylogenetic

tree the consensus sequence of the isolates comprising N4 were compared to reference strains for BVDV 2a and BVDV 2b by pairwise alignment using BLAST. The N4 consensus was 6% divergent from BVDV 2b and 7% divergent from BVDV 2a. This slight difference in sequence similarity is reminiscent of the ongoing debate of how much divergence defines a new subgenotype of BVDV 2. However the range of variation established by Vilcek et al. in 2004 is between 19% and 1% and the difference seen in this study between comparison of consensus sequences and established subtypes of BVDV 2 is 1%.

3.6. Further Research

Antigenic cross reactivity between popular BVDV 1a vaccine strains and field isolates of BVDV should be evaluated for protection against BVDV 1b in particular. The findings of this study and other recent studies indicate that BVD 1b is the most commonly occurring subtype of BVD1 in cattle and alpaca (Fulton *et al.*, 2002; Kim *et al.*, 2009) (this study). Vaccines for BVDV that protect against BVD 1b should be developed and made available for use in cattle. Based on evidence found in the literature killed vaccines containing BVD 1a cp and ncp strains did not provide adequate protection against BVD 1b ncp (NY-1) (Talens *et al.*, 1989) yet no available MLV vaccine and only one killed vaccine containing BVDV 1b ncp are available. No current literature is available regarding the efficacy of this single killed vaccine against BVD 1b in experimental vaccination trials.

Vaccine efficacy trials should be performed with BVDV 1b as a challenge strain. The trials should test the time frame of immunity provided by killed and live modified strains of BVDV 1b. Previous studies concluded that a likely complicating factor in the effectiveness of BVDV 1b vaccine in particular is that there may be a lag before induced immunity between BVDV 1a strains and BVDV 1b vaccine strains. This lapse in timing may be to blame for the increased numbers BVDV 1b infections and not BVDV 1a strains.

Is this a case of repeated and prolonged exposure? Studies that address the disproportionate occurrence of BVDV 1b in cattle should be performed focusing on the role of PI and TI animals in herds. A possible research question could be proposed: Is there something inherently different about BVDV 1b that increases its ability to infect fetal cattle and give rise to PI animals in pregnancy? If BVDV 1b were found to have increased tissue affinity for placental tissue then conclusions could be drawn for the role of BVDV 1b in PI animals. If BVDV 1b were found to have no preferential role in PI then is BVDV 1b a TI problem?

A BVDV vaccine for use in camelids should be developed. Currently all of the available vaccines for BVDV are labeled for use in cattle only and a BVDV vaccine has not been tested for efficacy in alpaca (Kentucky Llama and Alpaca Association 2009).

Sequence information regarding BVDV strain subtype used in this study was obtained from published studies for the establishment of subtypes using known BVDV strains. NCBI GenBank information regarding subtype was often not found in the description of BVDV sequences. A large database containing information regarding BVDV subtype for known strains should be compiled and made publically available.

Diagnostic detection and genotyping of positive samples as BVDV 1 or BVDV 2 is commonly practiced in veterinary diagnostic laboratories as a means of identifying and reporting occurrences of BVDV 2. Reporting the occurrences of BVDV 1 is not required by the USDA or OIE. Sequencing of all BVD viruses found by diagnostic detection is not commonly practiced due to the increased amount of labor, time and cost associated with sequencing in order to gain information that may not alter recommendations to the herd owner by the diagnostician. However, sequence data allow for subtyping of all BVDV found in veterinary samples thus allowing an additional level of analysis for use in epidemiological studies. Future understanding of the association of certain BVDV subtypes may reveal patterns in BVDV clinical signs. These data could be useful on two levels. First, sequence data used for subtyping at the laboratory level

could be submitted and archived in a larger database. Basic epidemiological information such as time of death (or diagnosis) and geographic location could provide additional insight into broad patterns of BVDV occurrence with very little change in laboratory procedures at the bench level. Second, sequence data gathered along with reproductive data from herd owners could provide robust data for the analysis of virulence and disease etiology of BVDV subtypes at the herd level. Dairy farms in Pennsylvania could be asked to participate in a study by mail survey along with a sampling regimen for BVDV through submission of either blood or ear notch samples. These two approaches (sequence/geography or sequence/reproductive records/vaccine) would show patterns of BVDV subtype occurrence and could lead to development of improved vaccines for BVDV as well raise awareness of BVDV prevention and control through public participation.

Additional studies involving necropsy specimens may be carried out by testing individual tissues for BVDV subtype and sequence variation within the animal. Sequence analysis could be used to show virus tissue affinity in both PI and TI animals by identifying virus load in a known amount of tissue by Real-Time RT-PCR quantification followed by sequence analysis of the 5'UTR of the BVD viruses recovered from different tissues within a single host.

The possibilities of unknown reservoirs for BVDV have been suggested. BVDV has been shown to be spread through an insect vector where flies that had contact with infected cattle were shown to carry viable BVDV particles and infect susceptible animals (Tarry et al 1991). Biting insects such as mosquitoes have also been implicated as a possible reservoir for BVDV (Dinter & Morein, 1990).

This study shows that subtype BVDV 1b is the predominant subtype of BVDV recovered from Pennsylvania cattle between 1997 and 2009 and that subtype BVDV 2b is likely present in the Pennsylvania cattle herd in proportions comparable to those reported for South American countries Brazil in particular. These findings are in agreement with published findings of BVDV subtype diversity in cattle throughout the United States with respect to the predominance of

subtype BVDV 1b. These findings show a higher than expected occurrence of BVDV 2b in the study population when compared to other studies of BVDV subtype occurrence in the United States. Further research concerning the origin of BVDV 2b in Pennsylvania may be possible through epidemiological analysis of point origin of cattle and their source herds in Pennsylvania.

BVDV subtyping may reveal a previously undescribed diversity of BVDV and is now becoming recognized as a classification system useful to herd owners, vaccine manufacturers and diagnosticians. The system of virus subtyping may increase as standard practice in diagnostic laboratories around the world. The next step in disease diagnosis along the lines of genomic evaluation may be whole genome sequencing. Virus typing by whole genome characterization may someday become standard practice in diagnostic laboratories. The only question is when. Technology exists today that allows for rapid sequencing of entire genomes. Although tests are very expensive to perform, the yield of raw data attained in a single test is staggering. Vaccine technology will no doubt advance as well. Perhaps viral genomic characterization and custom vaccine manufacture will meet to form a new approach in viral disease diagnosis, management and treatment. Until then in the practical sense of diagnostic testing, balancing test cost with diagnosis and treatment will remain an economic reality. In an ideal world the vaccine would be custom fit to a specific virus. Automation of laboratory testing, especially genomic based testing will continue to increase the likelihood that a whole genome snapshot will be available in molecular diagnostic tests not only of the pathogen but of the host as well. This approach will not only allow a diagnostician to match a viral genome with a treatment but also to predict the immunologic response of the host. The potential for the development of this approach to vaccine technology and disease treatment exists. The costs of implementing this type of testing and technology will be weighed against the benefit of the tests and ultimately will be decided by economics.

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